Environmental Stressors and Lipid Production by *Dunaliella* spp. I. Salinity

Stephanie M. Mixson Byrd[1,2]*, JoAnn M. Burkholder[2], and Paul V. Zimba[3]

[1]Center for Applied Aquatic Ecology, North Carolina State University, 620 Hutton Street – Suite 104, Raleigh, North Carolina 27606 U.S.A.

[2]Present Address: Medicago USA, 7 Triangle Drive, Research Triangle Park, NC 27709 U.S.A.

[3]Center for Coastal Studies, Texas A&M University Corpus Christi, Corpus Christi, TX 78412 U.S.A.

*Corresponding author. Tel.: 919-515-2726; fax: 919-513-3194.

E-mail address: jburk@ncsu.edu , steph.mixson@gmail.com

Abstract

Fourteen strains within four species of the marine chlorophyte genus, *Dunaliella* were assessed for their potential utility in sustainable biofuel production by tracking lipid production under salinity stress. A modified technique with Nile Red stain was used to screen cultures rapidly for the presence of neutral lipid content. Promising strains with visually high lipid content and high growth as cell production were selected to enhance lipid production using high salinity stress in short-term (sec to hr) and long-term (≥ 24 hr) bench-scale experiments (culture volume 0.1 to 3.5 L). These strains were also grown at mass culture scale (culture volume ~150 to 175 L). The difference in experimental scale was imposed because of the container effects shown for various algae, and in recognition of the importance of scale-up feasibility in harnessing algae for biofuel production. High salinity

stress generally resulted in maximal total fatty acid (FA) content (up to 65% by dry weight) in comparison to controls (~10-25% total FAs by dry weight). Glycerol production, a known mechanism of osmoregulation in *Dunaliella*, was measured in a short-term salinity stress experiment on a promising strain and found to increase significantly 30 min to 24 hr after exposure to high salinity. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to evaluate the relative expression of glyceryl-3-phosphate dehydrogenase (*GPDH*), one of the primary glycerol biosynthesis genes for glycerol production, during a short-term experiment with high salinity stress. *GPDH* was significantly expressed (≥ 2-fold when compared to the endogenous gene *ACTIN*) 30 min after exposure and continued to be expressed for 2 hr. In general, when cellular glycerol content was low, total FAs increased as an immediate or short-term response (30 sec to 30 min) to hyperosmotic stress. Responses were strain-specific and indicated both inter- and intraspecific variation. Overall, a simple high salinity adjustment significantly increased lipid production in selected strains of *Dunaliella* spp. The data suggest that these *Dunaliella* strains may incorporate a portion of the available glycerol as triacylglycerols (TAGs) or neutral lipids under short-term high salinity stress. Thus, glycerol acts not only as an osmolyte during hyperosmotic stress, but also provides the carbon structure to which FAs are covalently linked to form neutral lipids.

Keywords: *Dunaliella*, glycerol, *GPDH*, lipids, salinity, total fatty acids

1 Introduction

Agricultural crops including corn, sugar cane, and oil palm have been investigated as potential sources of biofuel, but they produce only a fraction of the oil per unit biomass in comparison to microalgae (Chisti, 2008; Schenk et al., 2008). Research about use of algal lipids for sustainable biofuel mostly has involved exposing algal cultures to a range of environmental stressors such as temperature, salinity, pH, light, and macronutrients (Hu et

al., 2008; Sharma et al., 2012; Valenzuela et al., 2013). The halotolerant saltwater microalgae *Dunaliella* spp. (Chlorophyta, Chlorophyceae) accumulate neutral lipids in response to various environmental stressors (Roessler, 1990; Tagaki et al., 2006; Ben-Amotz et al., 2009; Yao et al., 2016) and also have high growth rates (as cell production), making them ideal candidates for mass culture as a renewable biofuel source (Hu et al., 2008; Rodolfi et al., 2009; Araujo et al., 2011; and references therein). These species are halotolerant, able to tolerate a wide range of salinities from < 5 to full saturation (Ginzburg, 1987; Avron and Ben-Amotz, 1992; Ben-Amotz et al., 2009). In addition to not requiring freshwater for growth, this genus lacks a rigid cell wall (Ben-Amotz et al., 2009; Graham et al., 2016), which facilitates lipid extraction. As a result of their tolerance to high salt, *Dunaliella* species can more easily outcompete potential bacterial contaminants/competitors (Avron and Ben-Amotz, 1992; Hu et al., 2008).

Under hyperosmotic stress when external salinity increases relative to the cellular osmotic pressure, *Dunaliella* can accumulate glycerol (Ben-Amotz and Avron, 1973; Chitlaru and Pick, 1991; Ben-Amotz et al., 2009; Chen and Jiang, 2009) or fatty acids to high levels (Azachi et al., 2002; Takagi et al., 2006). Depending on the requirements of the cell, glycerol can either be synthesized or broken down into additional products such as starch, sugar, or pyruvate (Taiz and Zeiger, 2006; Chen and Jiang, 2009; Shariati and Hadi, 2011) (Fig. 1). Glycerol is produced by CO_2 fixation or the degradation of starch (Avron and Ben-Amotz, 1992; Ben-Amotz et al., 2009). Glycerol biosynthesis takes place primarily in the chloroplast, but also in the cytosol. Four enzymes are required for the inter-conversion of dihydroxyacetone phosphate (DHAP) to glycerol, including a key enzyme glycerol-3-phosphate dehydrogenase (*GPDH*) (Ben-Amotz et al., 2009; Chen and Jiang, 2009). This

gene catalyzes a reversible step in converting dihydroxyacetone phosphate (DHAP) to the intermediate product of glycerol-3-phosphate (G3P). G3P is then converted to the final glycerol product by the enzyme glycerol phosphate isomerase (*GPI*) (He et al., 2007; Chen and Jiang, 2009) (Fig. 1). Metabolism of glycerol depends upon whether the cell is actively growing and requires energy for growth and division, or the cell is in stationary phase or senescence, during which neutral lipids can be produced. In addition, glycerol provides the carbon structure or "backbone" for triacylglycerols (TAGs), formed by the condensation of glycerol with three fatty acid (FA) molecules (Miner and Dalton, 1953). Thus, it is assumed that when neutral lipids are produced under conditions such as nutrient limitation (Chen et al., 2011), pH stress (Gardner et al., 2012; Mixson et al., submitted), and/or salinity stress (Takagi et al., 2006), a proportion of the glycerol pool will decrease as TAGs form from the incorporation of available glycerol. FA molecules may be covalently linked to some of the excess glycerol produced in response to hyperosmotic shock to form TAGs.

Species within the genus *Dunaliella* react to osmotic stress through an *immediate response* (30 sec to $\leq$ 5 min) wherein cell size, shape, structure, and ion concentration change; a *short-term response* (up to 2-3 hr) whereby the osmotic pressure between the outside environment and inside the cell is balanced by regulating the glycerol concentration; and a *long-term response* ($\geq$ 24 hr) which includes stress-induced gene expression and accumulation of salt-induced proteins (Chen and Jiang, 2009). These responses take into consideration immediate and short-term acclimation to changes in the environment as well as epigenetic adaptation, whereby gene expression can be up- or down-regulated in response to a particular stressor (Lakeman et al., 2009; Sahu et al., 2013). In this research, the optimum hypo- or hyper-osmotic salinity stress was assessed for maximizing lipid production by

selected strains of *Dunaliella*. Cells undergo hypo-osmotic stress when external salinity decreases, and they eliminate glycerol to balance the cellular osmotic potential. Therefore, it was expected that *Dunaliella* would not produce neutral lipids during hypo-osmotic shock (Ben-Amotz et al., 2009) due to the lack of available glycerol, required for the formation of TAGs.

A time course experiment with short sampling intervals was also completed, using a subset of selected strains, to determine when the highest production of total FAs and glycerol production initially occur under high salinity stress. The data were helpful in guiding quantitative PCR, which was used in a time course experiment to analyze expression of the glycerol biosynthesis gene, *GPDH*. It was expected that (i) total FAs would increase rapidly or as a short-term response under high salinity stress; (ii) as total free glycerol increased, there would be a corresponding expression of *GPDH*; and (iii) a proportion of the available glycerol would be incorporated by the cells into TAGs.

2 Materials and Methods

2.1 Strain Purification and Identification

Until recently, the taxonomy of the Chlorophyceae was based on morphological characteristics under light microscopy along with biochemical characteristics (Ben-Amotz et al., 2009). Use of only physiological criteria to distinguish among different strains of *Dunaliella* can be unreliable, however, as well as difficult to interpret (Ben-Amotz et al., 2009; Assunção et al., 2012). As a revision to the genus *Dunaliella*, Massjuk (1973) identified 28 species, but many strains have been misnamed and new species names have been unnecessarily introduced (Olmos-Soto et al., 2002). Additionally, complications arise

126 due to the enormous intraspecific variability of strains within a given species (Gómez and
127 González, 2004; Assunção et al., 2012).

128 Fourteen strains of *Dunaliella* were obtained from the American Type Culture
129 Collection (ATCC), the Culture Collection of Algae and Protozoa (CCAP), the Provasoli-
130 Guillard National Center for Marine Algae and Microbiota (NCMA, formerly the National
131 Center of Marine Phytoplankton), and the Culture Collection of Algae at the University of
132 Texas at Austin (UTEX) (Table 1, Fig. 2a-n). These strains were labeled by the culture
133 collections within five species of *Dunaliella*, but culture collections and GenBank are well
134 known to contain misidentified species, misinformation that has been perpetuated in the
135 literature (Borowitzka and Siva, 2007). In addition, cultures from commercial collections
136 commonly are contaminated with other microorganisms. Therefore, the 14 strains were first
137 purified before conducting salinity stress experiments, and then the taxonomy of the
138 *Dunaliella* strains was checked by characterizing their ITS region.

139 Strains were purified using a combination of differential centrifugation and flow
140 cytometry (Beckman Coulter® EPICS Altra, NCSU) to remove bacterial contamination
141 (Guillard, 2005; Kawachi and Noël, 2005). Cultures were plated on 1% Agar M (Sigma®,
142 Sigma Aldrich, Carlsbad, California, USA) or 1% Phytoblend (Caisson Laboratories, Logan,
143 Utah, USA) to promote algal growth or bacterial growth, respectively. Subsequent plating
144 was completed aseptically on a monthly basis until each strain was purified. Stock culture of
145 each strain (n = 3) was grown under the additional conditions of 23˚C and a light intensity of
146 ~180 µE m^{-2} sec^{-1} (light source, ProLume® compact fluorescence bulbs) under a 12-hr : 12-
147 hr light : dark (L:D) photoperiod.

Total DNA was extracted from 50 mL of exponentially growing cells (~10^6 cells/mL). Cells were first pelleted (3,000 RPM, 10 min) and then DNA was extracted using a modified CTAB extraction method (Stewart and Via, 1993; Walker et al., 2005) for each of the 14 strains. Following Walker et al. (2005), an equal volume of Solution A (1% Sarcosine, 0.8 M NaCl, 0.22 M Tris-HCl - pH7.8, 22 mM EDTA, 0.8% cetyl trimethyl ammonium bromide (CTAB), 0.14 M mannitol) and chloroform : isoamyl alcohol (Sigma®; 24:1) was added to the samples, and the mixture incubated at 65˚C for 10 min. Samples were centrifuged (14000 RPM, 5 min), the aqueous phase collected, and RNaseA (Qiagen, Venlo, The Netherlands; 1 mg/mL, final concentration) was added. After incubation at 37˚C for 20 min, an equal volume of chloroform : isoamyl alcohol (24:1) was added, the samples centrifuged, and the supernatant collected. An equal volume of isopropanol (Sigma®) was added to precipitate the DNA and samples were centrifuged at maximum speed. The DNA was re-suspended in TE (Fisher®; 10:1) buffer (Fisher Scientific, Pittsburgh, Pennsylvania, USA).

ITS1 and ITS2 are non-coding introns between the 18S, 5.8S, and 26S rDNA portions (Müller et al., 2007). Genetic mutations occur naturally within a population and can be used to resolve intraspecific and interspecific relationships within and among *Dunaliella* species. The ITS region is conserved; however, differences can be found in the sequence indicating intraspecific variation among strains (Gómez and González, 2004; Assunção et al., 2012). The ITS2 has evolutionarily conserved secondary structure and a frequency of mutation that allows for strain level differentiation (Keller et al., 2010; Assunção et al., 2012). In this study, amplification of the ITS region (González et al., 1998) was completed using a GeneMate Genius, with ITS primer set sequences (Promega; the original source of the primers was Goff et al., 1994). The 5' to 3' sequence for (forward) primer TW81 was

GGGATCCTTTCCGTAGGTGAACCTGC, and for (reverse) primer AB28 was GGGATCCATATGCTTAAGTTCAGCGGGT. For ITS amplification, the protocol described in González et al. (1998) was used. Thermocycler conditions were as follows: five cycles of 90˚C for 1 min, 50˚C for 2 min followed by one cycle of 68˚C for 1 min, and 30 cycles of 90˚C for 1 min, 60˚C for 1 min, 68˚C for 1 min, and a final annealing stage of 68˚C for 10 min. ITS product was confirmed by 1% agarose (Sigma®) gel electrophoresis and visualized with GelStar® on a Gel Doc XR + UV transilluminator using Image Lab Software.

PCR products were purified using a Qiagen QIAquick PCR purification kit, following manufacturer's instructions. Purified products were prepared according to GENEWIZ, Inc. instructions and sequenced (GENEWIZ, Inc.). Consensus sequences were designed by aligning the forward and reverse strands and comparing all nucleotides to the original chromatograms. Clustal Omega (1.1.0) was used to align multiple sequences using default parameters. Lastly, MEGA5 (maximum likelihood statistical method; 100 bootstrap replications; nearest-neighbor-interchange) was used to create a phylogenetic tree for the ITS2 region (Tamura et al., 2011). Final analyses were completed by comparing the strain sequences to available sequences on GenBank. (http://www.ncbi.nlm.nih.gov/genbank/) (Table 2).

The ITS2 sequence data for each of the 14 strains indicated that they actually fell within four distinct species (clades) (Fig. 3), and that 10 of the 14 strains had been labeled to species incorrectly (Tables 1 and 2). In addition, the strain sent to us by the UTEX culture collection in 2009, labeled *Dunaliella salina* 1644, was determined by from ITS2 sequence data to be a unique strain of *Dunaliella viridis* that evidently was a culture contaminant in the UTEX

collection (Mixson, 2013). It was unique from all of the other *Dunaliella* strains tested in major features including cell size (very small), population growth as cell production (very rapid), sustained growth in *continuous* light, and high lipid production *throughout* the cell cycle. Based on that information, the strain was not simply a contaminant from the other tested *Dunaliella* cultures. Strain UTEX 1644 was ordered in 2013 from the UTEX collection by another laboratory (Srirangan et al. 2015), and was confirmed as *D. salina* rather than this unique strain. Thus, the unique strain sent to us by the UTEX collection in 2009 was a contaminant that can no longer be obtained under the number, UTEX 1644. The research revealed that this strain is of special interest for its growth and lipid production, so it was deposited it at the UTEX collection (*D. viridis* strain UTEX ZZ1150). The 14 strains below are referred to by the species names indicated from these ITS2 sequence data, along with the original strain numbers except for reference to *D. viridis* strain UTEX ZZ1150.

2.2 Culturing and Selection of Strains for Experiments

All strains were first cultured in the media used by the commercial culture facilities of origin (Table 1), except for *D. salina* ATCC 30861 which was provided in L1-Si medium (Guillard and Hargraves, 1993) at salinity 105 (adjusted using artificial salts). The planned salinity stress experiments required use of the same medium. Preliminary work indicated that the *Dunaliella* strains grew well in Erdschreiber's (Føyn, 1934), Artificial Seawater (ASW, made using f/2-Si nutrients - Guillard, 1975; Tompkins et al., 1995), and L1-Si media, at salinity 30 or 60 depending on the strain. Erdschreiber's and ASW media contained soil water with undefined constituents, and nutrient components of f/2-Si were similar to those of L1-Si. Therefore, L1-Si was selected for use in this study. Once growth was established for strains in their source media (salinity 30 or 60), each culture was transferred into a series of

218 increasing proportions of L1-Si and the source medium (after Lorenz et al., 2005) until the
219 cells finally were acclimated to 100% L1-Si medium. Cell production for each strain was
220 compared in its source medium versus L1-Si medium, and all strains attained comparable or
221 higher maximum cell production in L1-Si (Table 3). In a second series of transfers, cultures
222 were adjusted using NaCl by salinity ± 5 to 10 for each adjustment (YSI 3200 Conductivity
223 Instrument, Yellow Springs, Ohio, U.S.A.), and tested for population growth rates (as cell
224 production) to determine the optimal salinity for each strain (Table 1). An analogous
225 approach was used to identify the optimum pH for each strain, using incremental changes of
226 0.1 pH unit (PerpHecT LogR Meter 350, Boston, Massachusetts, U.S.A.) from an initial pH
227 of 7.5 to 8.5.

228 Population growth rates (as cell production) were assessed by growing each strain in
229 100 mL volume (n = 3) as unialgal culture until late senescence (~35 days). Subsamples were
230 taken between 0800 and 0900 every other day, preserved in acidic Lugol's solution (1% final
231 concentration; Vollenweider et al., 1974), and quantified within 1-2 days using Palmer-
232 Maloney chambers (Wetzel and Likens, 2010) under light microscopy at 200x magnification
233 using an Olympus BH-2 light microscope (Olympus Corporation, Center Valley,
234 Pennsylvania, U.S.A.).

235 The mean biovolume for each strain was also determined as follows: The cells were
236 immobilized using 1.3% Type V agarose (Sigma Aldrich). Two drops of agarose and two
237 drops of culture were placed in the center of a warmed slide and gently mixed using a pipette.
238 After the agarose had solidified, at least 30 micrographs of each *Dunaliella* strain were taken
239 at 600x (No. 1.5 25mm^{-2} coverslip, Corning®), using an Olympus AX70 microscope and an

Olympus DP70 camera. Biovolumes were calculated using the formula for a prolate spheroid (Hillebrand et al., 1999) (Table 1):

$$V = \left(\frac{\pi}{6} * d^2 * h\right) \quad (1)$$

wherein V ≡ biovolume (μm^3), d ≡ diameter (μm), and h ≡ height (μm).

The 14 strains were also screened visually for high neutral lipid content using the fluorescent Nile Red dye (NR, 9-diethylamino-5-benzo[α]phenoxazinone; Sigma Aldrich). NR preferentially stains neutral lipids (triacylglycerides, TAGs), and has been used as a rapid screening technique to assess qualitatively the neutral lipid content of microalgal cells (Cooksey et al., 1987; Lee et al., 1998; Chen et al., 2009; Yao et al., 2016). The technique was optimized for use with *Dunaliella* (Mixson, 2013): Briefly, 10 μL NR (0.625 μg/mL dissolved in 100% methanol, MeOH) was added to slightly heated slides containing 2 drops of culture sample immobilized on 1.3% Type V agarose (Sigma Aldrich). An Olympus AX70 research light microscope and Olympus DP70 camera system, or a Zeiss LSM 710 confocal microscope (excitation at 488 nm, emission at 545-665 nm) was used to photograph NR-stained cells (Fig. 4).

This rapid screening technique was used to determine when selected strains (*D. tertiolecta* strains UTEX 999, UTEX 1000, and CCAP 19/9; and *D. viridis* UTEX ZZ1150) had maximal neutral lipid content, considering both the growth cycle and the light regime. Samples for these tests were collected every other day at 1000 and 1600 hr for cell counts and fluorescence microscopy of NR-stained cells, until cultures were senescent. Samples then were taken at 0400, 1000, 1600, and 2200 hr for analysis of neutral lipid content using the NR rapid screening technique. All tested strains except *D. viridis* UTEX ZZ1150 had

maximal neutral lipid content during senescence, and senescent populations had comparable content of neutral lipids throughout the light period. In contrast, rapid screening indicated that the unique strain *D. viridis* UTEX ZZ1150 had high lipid content during active growth as well as senescence. These observations guided sampling for the salinity stress experiments: The *Dunaliella* populations were sampled for total FA content when the populations had reached early onset of senescence, and sampling was conducted between 0800 and 0900 hr.

The data for cell production and rapid screening of neutral lipid content were used to select seven strains for long-term salinity stress experiments (100 mL volume). These strains had high maximal cell production and/or visually high amounts of neutral lipids, ideally both features. Growth curves (as cell production) were generated for each strain (Fig. 5).

Of the seven strains, the four with highest lipid production in the long-term salinity stress experiment were further tested for their immediate and short-term responses to increased salinity. Strain *D. viridis* UTEX ZZ1150 additionally also tested for glycerol production in these short-term experiments. Mass culture of *Dunaliella* has been commercially successful for β-carotene production (Olmos-Soto et al., 2002). Here, two strains (*D. tertiolecta* UTEX 999 and *D. viridis* UTEX ZZ1150) were also grown in mass culture (~150 to 175 L volume) to check feasibility of scale-up.

2.3 Total FAs

The historically used, gravimetric solvent-based extraction methods of Folch (1957) and Bligh and Dyer (1959) are time-consuming (6-8 hr required to process six samples), but also require large amounts of solvents per sample, a methylation step after extraction, and a final step of drying with nitrogen gas. With each step, the potential for error in analysis of

relatively small samples becomes greater. A third method considered, direct transesterification (DT) (Griffiths et al., 2010), combines lipid extraction and purification steps, requires less than one-third the volume of chemicals per sample, and enables processing of at least 24 samples within 4 hr. In DT, extraction and methylation are combined in a one-step procedure, thus reducing potential sample loss, and all steps are completed in one test tube. In addition, the final layer of hexane containing the neutral lipids can be directly injected into the GC for analysis of total FAs. In analysis of *Dunaliella* samples using these three methods, DT yielded less variability among samples and comparable amounts of total FAs (23.03 $\pm$ 2.38 mg/g, means $\pm$ 1 standard deviation [SD], n = 3) as the Folch, Bligh and Dyer, and Bligh and Dyer + salt procedures (22.86 $\pm$ 4.56 mg/g, 22.89 $\pm$ 13.55 mg/g, and 22.11 $\pm$ 10.78 mg/g, respectively). Similarly, Griffiths et al. (2010) found that DT was the more convenient method in terms of total volumes of solvents used and time required, and it was also more accurate than solvent-based extraction methods in quantifying microalgal total fatty acids (FAs ≡ fatty acid methyl esters) content.

DT was performed to convert saponifiable lipids to total FAs, which were then quantified using gas chromatography (GC) and flame ionization detector (FID). As indicated above, this technique offers several important advantages over conventional solvent-based extractions. DT combines lipid extraction and purification steps in one tube, thus minimizing sample loss, and can be completed on the small sample sizes needed in this research. The combination of adding basic and acidic catalysts (in this study, KOH and BF_3, respectively) allows for samples to be processed even if the samples contain small amounts of water (Griffiths et al., 2010; Arias-Forero et al., 2013), which was important because these samples were not dried before processing.

311 Briefly, 0.5 M methanolic KOH was added to each sample, homogenized, and heated to
312 85˚C. BF_3 (14% in MeOH) was added and the samples were reheated to 85˚C. Then, equal
313 volumes of water and hexane were added and the samples were allowed to separate into
314 layers by density. The lighter hexane layer containing the neutral lipids was removed and
315 analyzed with a GC HP5890 series II GC equipped with a FID (Hewlett Packard, refurbished
316 by Primera Scientific LLC, Princeton, New Jersey, U.S.A.) and a 7673A autosampler (Alpha
317 Omega Technologies, Inc., New Jersey, U.S.A.). Separation was achieved in an Rtx-2330
318 capillary column (Alpha Omega Technologies, Inc., refurbished by Primera Scientific LLC,
319 Princeton, New Jersey, U.S.A.). The temperature was programmed to include an initial 3 min
320 at 60˚C, and then was increased to 230˚C at a rate of 4˚C per min. Both injector and detector
321 temperatures were set at 265˚C. Injections were performed under the splitless mode
322 (Sparkman et al., 2011). Data acquisition and analysis were completed using GC
323 Chemstation Rev. A.08.03 (847) software. Calculations for total FAs were completed using
324 the relative response factor (RRF) method (Sparkman et al., 2011; L. Dean, USDA, Raleigh
325 NC, personal communication), and area ratios were compared to the internal standard (KEL-
326 FIM-FAME-5 Mixture, Matreya, LLC, Pennsylvania, U.S.A.).

327 2.4 Wet weight : dry weight determination

328 The wet weight : dry weight ratio is of paramount importance in studies assessing
329 lipid production potential based on relatively small cell mass of microalgae (Zhu and Lee ,
330 1997; Mata et al., 2010; Schlagermann et al., 2012; Coons et al., 2014). The optimal method
331 for determining a wet-weight-to-dry-weight conversion factor for three strains of *Dunaliella*
332 (*D. tertiolecta* UTEX 999, *D. tertiolecta* UTEX 1000, and *D. viridis* UTEX ZZ1150) was
333 found to be the use of ammonium formate (0.5 M) wash or KF titration. Each filter held ~250

mL of dense culture before becoming clogged. Using filtered medium or no wash yielded a 90% conversion factor. The higher dry weight yielded from samples washed with filtered medium was due to NaCl being retained on the filter paper or in intercellular spaces. Use of DI as a wash yielded a 98% conversion factor. The significantly lower weight yielded by samples rinsed with DI occurred because of cell lysis due to the severe difference in osmotic potential. Both 0.5 M ammonium formate and KF titration yielded a 95% wet-weight-to-dry-weight conversion factor. Zhu and Lee (1997) suggested using ammonium bicarbonate as an alternative to ammonium formate because this chemical is readily available and more cost-effective. Both solutions are isotonic and have been demonstrated as effective washing agents for marine microalgae samples (Zhu and Lee, 1997). KF is a specific determination method because only water content is calculated. This method is highly reproducible and precise over a wide range of concentrations from <1% to 100% (Bruttel and Schlink, 2003). The calculation used for converting total FAs (wet weight basis) to total FAs (dry weight basis) was:

$$\% \text{ Total FAs (dry weight)} = [(\% \text{ Total FAs (wet weight)})/5] \; . \qquad (2)$$

Percent total FAs (by wet weight) was calculated from the GC software Chemstation Rev. A.08.03 (847) using the RRF method. The "5" in the equation represents the wet-weight-to-dry-weight conversion factor (100 – 95) as previously determined.

2.5 Long-term salinity stress

Preliminary experiments, completed to determine appropriate intervals for the salinity stress experiments, tested increasing or decreasing salinity shifts of 5, 10, 15, 25, 30, and 50 on selected *Dunaliella* strains. The smaller levels of change (increments of salinity 5

and 10) did not appear to stress the cells based on cell size and shape, and cell production. The upper limit was an increase of 50; when cells were exposed to a salinity of 50 higher than the previous salinity, cell production ceased and after 7 days there was no recovery from the hyperosmotic shock. When exposed to a salinity of 15 or 30 above the previous salinity, there was a slight lag in cell production over several days. Based on these data, in the long-term salinity stress experiments, a series of salinity shifts of 15 was imposed, with shifts separated by 7-day intervals to allow the populations to slowly acclimate to increasingly higher or lower salinity levels.

Seven selected strains (*Dunaliella tertiolecta* strains CCAP 19/9, CCAP 19/24, CCAP 19/26, UTEX 999, and UTEX 1000; *D. pseudosalina* CCAP 19/18; and *D. viridis* UTEX ZZ1150; Table 1) were separately grown in replicate cultures (n = 3) in 100 mL volumes for 14 days until the onset of early senescence. Small-volume samples (~1 mL) were taken at 3-day intervals and preserved in acidic Lugol's solution for cell counts as described above. After the first week, 5 mL of exponentially-growing culture were inoculated into 100 mL of fresh medium that was higher or lower in salinity (by 15) than the previous medium. This procedure was repeated with increasing or decreasing salinity until the cultures attained one-third to one-half of the maximal cell production of controls (maintained at the initial, optimal salinity for each strain). The salinity at this point was interpreted as the upper or lower tolerance limit of the strain to hyper- or hypo-osmotic salinity stress, respectively. After the onset of early senescence at each salinity, cultures were centrifuged (3,000 RPM, 10 min) and the pellets were frozen immediately at -80˚C until total FA analysis was completed (within 7-10 days for all experiments; L. Dean, U.S. Department of Agriculture, Raleigh North Carolina, USA, personal communication).

382 2.6 Short-term stress from increasing salinity

383 Four selected strains (*Dunaliella tertiolecta* strains CCAP 19/9, CCAP 19/24, and
384 UTEX 999, and *D. viridis* UTEX ZZ1150; n = 3) were each grown in unialgal culture at ~3.5
385 L volume under optimal salinity and pH, as above. When cultures reached senescence, the
386 culture volume of the three replicate flasks was evenly distributed into seven sterile 50-mL
387 tubes by centrifuging (3,000 RPM, 10 min). Each 50-mL aliquot was centrifuged repeatedly
388 until a total of ~500 mL of culture was obtained within each tube. Two salinity stress
389 treatments were imposed by adding L1-Si medium at a salinity +15 or +30, adjusted with
390 NaCl (n = 3 for each strain at each salinity). Control pellets were re-suspended with 50 mL of
391 L1-Si medium at the initial, optimal salinity (30 or 60) for each strain. Treatment pellets were
392 resuspended with 50 mL of L1-Si at a salinity +15 above the control salinity. A shift of +30
393 above the control salinity was also tested to assess the effect of more severe short-term
394 salinity stress on cell production and total FA content. After resuspension, all replicates were
395 subsampled for cell counts and NR rapid screening of neutral lipid content at 30 sec, 5 min,
396 30 min, 1 hr, 2 hr, and 24 hr after the salinity adjustments. Re-suspended samples
397 (concentrated from ~500 mL of culture) were centrifuged (3,000 RPM, 10 min) and the
398 pellets were frozen immediately at -80˚C until FA analysis was completed.

399
400 2.7 Total free glycerol

401 A short-term salinity stress experiment was completed using *D. viridis* UTEX ZZ1150.
402 This experiment deviated from the above approach (Section 2.6) only in that the culture was
403 divided into 15-mL centrifuge tubes, each of which contained a pellet concentrated from
404 ~175 mL of stock culture (n = 3). Each pellet was then re-suspended in 15 mL of fresh
405 medium within a sterile centrifuge tube, either at the control salinity (60, for this strain) or at

406 salinity 90. After each exposure period, a set of tubes was centrifuged and the supernatant
407 was saved in a new, sterile 15-mL centrifuge tube. Both the pellet and its corresponding
408 supernatant were quantified for total free glycerol content. Pelleted samples were each
409 dispersed into 1 mL of internal standard mix consisting of cellobiose in water (Patee et al.,
410 2000). From this solution, a 50 µL aliquot was diluted with 2 mL water. Samples of the
411 supernatant (1 mL each) were spiked with 50 µL of the internal standard and diluted to 2 mL
412 with deionized water. Total free glycerol was analyzed as described by Patee et al. (2000);
413 the data were reported as mg/g for pellets and mg/mL for supernatants.
414
415 2.8 Time course of glycerol production under hyper-osmotic stress
416 A short-term salinity experiment as above (Section 2.6) was completed using *D.*
417 *viridis* UTEX ZZ1150. Centrifuged pellets re-suspended into 50 mL of culture medium at the
418 control salinity of 60 or at the stress salinity of 90. After re-suspension, replicates were
419 centrifuged (3,000 RPM, 10 min, 4˚C) and frozen immediately at -80˚C until RNA
420 extractions were completed (within 7-10 days). Total RNA was isolated with a RNeasy Plant
421 Mini Kit (Qiagen, Maryland) following manufacturer's instructions. The RNA was
422 visualized by gel electrophoresis (1% agarose) to evaluate its integrity. A NanoDrop^{TM} ND-
423 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, U.S.A.) was used
424 to measure the total RNA concentration (ng/µL). High-quality RNA (~ 500 ng) was prepared
425 using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, California,
426 U.S.A.) to synthesize first-strand cDNA (complementary DNA), following manufacturer's
427 instructions using an Oligo$(dT)_{20}$ primer.
428 The endogenous gene *ACTIN* was selected as the housekeeping gene for these
429 experiments because it expresses at the same level regardless of physiological stress (He et

al., 2007; Chen et al., 2011). Forward and reverse primer pairs for the two genes (*ACTIN* and *GPDH*) were initially designed following Chen et al. (2011 – for *ACTIN*; GenBank accession no. AF163669.2; He et al. (2007), Chen et al. (2011 – for *GPDH*; GenBank accession no. EU624407.1, and following criterion guidelines for primer design (Klatte and Bauer, 2009, and methods therein). The primer sequences were confirmed using the online program Primer3 (Rozen and Skaletsky, 1998), and then were slightly modified with MacVector (MacVector, Inc. V. 12.6 2013) and Amplify3 (Amplify3 V.3.1.4 2005) to ensure that a single 153-bp product would be amplified (Table 4). Thermocycler conditions were as follows: an initial heating cycle of 95˚C for 30 sec; 30 cycles of 95˚C for 5 sec; 54˚C for 34 sec; 68˚C for 5 sec; and a final annealing stage of 68˚C for 7 min. A single 153-bp product was confirmed by gel electrophoresis (1% agarose) and sequencing (GENEWIZ, Inc., South Plainfield, New Jersey, U.S.A.).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was run on an Applied Biosystems StepOnePlus^{TM} real-time qPCR system following manufacturer's instructions (Applied Biosystems, Foster City, California, U.S.A.). A master mix was prepared for each cDNA (control, 30 sec, 5 min, 30 min, 1 hr, 2 hr, 24 hr, and non-template control or NTC). For a 20-µL total volume, 10 µL of SYBR® Select Master Mix (Invitrogen), 6 µL of the primer pair, 3 µL DEPC-treated water, and 1 µL cDNA were prepared per well for a 96-well plate (n = 3) (Invitrogen). Primer pairs with each set of cDNA were run in triplicate. Relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Edwards et al., 2004) relative to the endogenous gene *ACTIN*.

2.9 Mass Cultures

453 The mass culturing facility consisted of a series of growth tubes, each 1.21 m height
454 x 30 cm diameter, made of lightweight 0.1-cm polymer fiberglass, providing ~90%
455 transparency. Each tube held a maximum of ~175 L. Unless otherwise specified, cultures
456 were grown under a 12:12 L:D photoperiod. The light source consisted of light banks
457 (Standard Utilitech fluorescent lights) that provided ~160 to 180 µE/m^2/sec for each tube.
458 The temperature for these experiments was maintained at 23 ± 1^{o}C. Tubes were sampled
459 daily between 0800 and 0900 hr for temperature, pH (Oakton Waterproof pH Testr 30 Pocket
460 pH Tester, Oakton Instruments, Vernon Hills, Illinois, U.S.A.), and cell counts (Section 2.1).
461 In preparation for mass culturing, tubes were filled with ~150 L deionized water
462 adjusted to the desired salinity (30 or 60) (Hydrolab MiniSonde 4, Hach Company, Loveland,
463 Colorado, U.S.A.) and pH (8.3-8.4) with Coralife® salts (Franklin, Wisconsin, U.S.A.) +
464 ~19.35 mL each of parts ProLine® A/B (F/2; Aquatic Eco-Systems, Apopka, Florida,
465 U.S.A.) nutrient mixtures (Guillard 1975). Stock culture was grown by inoculating ~3 L
466 volume (salinity 30 or 60, adjusted with Coralife® salts + L1-Si nutrients) with ~300 mL of
467 culture (i.e. a 10% inoculum) under the growth conditions described above (Section 2.1).
468 After the stock culture reached late exponential growth phase (as determined by cell counts –
469 see Section 2.1), it was inoculated to a freshly prepared mass culture tube (~3.5 L stock
470 culture in ~150 L prepared medium). When this inoculum tube reached late exponential
471 growth (typically within 7-10 days), ~15 L were dispensed into each freshly prepared mass
472 culture tube that was filled with ~150 L culture medium as described above. Thus, the
473 maximum volume for any given tube was ~165 L including culture. The total number of
474 tubes prepared varied by experiment. Cultures of *Dunaliella* were grown to a density of 10^6

475 (rarely 10^7) cells/mL within 7-10 days depending on the strain and the experimental
476 conditions.
477
478 2.10 Statistics
479 Data were analyzed by one-way ANOVA with repeated measures (SAS v.9.2 – SAS
480 Institute, Inc., 2009). Treatment effects were considered significant at $p \leq 0.05$ (one-sided).
481 Type 3 Tests of Fixed Effects were completed to ensure that controls were not significantly
482 different, and indicated that replicates were similar in all experiments.
483
484 **3 Results**
485 3.1 FA composition
486 Total FAs were analyzed for constituent FAs for each of the seven strains tested
487 (Table 5). In general, the FA composition of the strains was similar, regardless of their
488 optimal salinity (30 or 60). Strain *D. viridis* UTEX ZZ1150 contained a small amount of C
489 17:0 (0.42% of the total), however, which was not observed in the other six strains (Table 5).
490 In addition, *D. pseudosalina* CCAP 19/18 lacked the FAs C18:2, cis9,12 and C18:3,
491 cis6,9,12, which were present in the other strains. Higher proportions of C18:1, cis9 and
492 C18:2, cis9,12 (~7.6 and 7.2%, respectively) were found in *D. viridis* UTEX ZZ1150 and *D.*
493 *tertiolecta* CCAP 19/24 when compared to the other four *D. tertiolecta* strains (CCAP 19/9,
494 CCAP 19/26, UTEX 999, and UTEX 1000), which contained only 3.7 to 5.6% of these FAs
495 (Table 5). *D. tertiolecta* CCAP 19/24 had an optimal salinity of 60, compared to the other *D.*
496 *tertiolecta* strains tested that had an optimal salinity of 30. The four strains tested under
497 short-term salinity stress (*D. tertiolecta* strains CCAP 19/9, CCAP 19/24, and UTEX 999,

498 and *D. viridis* UTEX ZZ1150) all exhibited similar proportions of FAs when compared to
499 their FA composition under optimal salinity conditions (Table 5).

500 The four *D. tertiolecta* strains (CCAP 19/9, CCAP 19/26, UTEX 999, and UTEX
501 1000) behaved similarly in response to long-term salinity stress with regard to percent total
502 FA composition. In general, these three strains decreased in C16:0 (from ~15% to 12%)
503 when exposed to high salinities (salinity 30 to 120). Proportions of C18:1 cis9 also decreased
504 in these strains (from 7% to 1.5%) when exposed to high salinities. The FA C16:3, cis
505 increased from ~12% to 19% under the same high salinity conditions.

506 Strains *D. viridis* UTEX ZZ1150 and *D. tertiolecta* CCAP 19/24 increased in the FA
507 C18:3, cis9,12,15 when exposed to salinities 60 to 150 (from ~25% to 30% and from ~31%
508 to 38%, respectively). Both strains also exhibited an increase in proportions of the FA C18:2
509 cis9,12 under high salinity stress of 60 to 150, but *D. viridis* UTEX ZZ1150 yielded higher
510 proportions (from ~11 to 13%) when compared to *D. tertiolecta* CCAP 19/24 (from ~3% to
511 5%).

512
513 3.4.b Long-term salinity stress in bench-scale experiments

514 All seven strains attained highest growth as cell production at the control salinity (30
515 or 60) which had been tested as optimal for each strain. Maximal population density among
516 strains ranged from 2 to 8 x 10^6 cells/mL (Table 1, Fig. 5). Within this range, the strain with
517 the smallest mean cell size, *D. viridis* UTEX ZZ1150 (biovolume 74 ± 5 μm^3), had the highest
518 maximal density, whereas the strain with the largest cells, *D. pseudosalina* CCAP 19/18
519 (biovolume 215 ±10 μm^3), had the lowest maximal density (see Table 1 for strain biovolume
520 and cell production). All strains except *D. pseudosalina* CCAP 19/18 were able to survive in
521 salinities of 90 higher than the control salinity; the exception survived at a salinity of 105,

522 only 45 higher than the control salinity of 60 for that strain. Sample weights for each strain
523 remained constant over the course of the experiment (~ 0.1 g). DT and subsequent analysis
524 was sensitive and accurate at these weights. Thus, while cell production varied among the
525 strains, total biomass remained consistent.

526 Total FA content significantly increased under high salinity stress in comparison to
527 the total FA content of controls for four of the five *D. tertiolecta* strains, including CCAP
528 19/9 (Fig. 6A), CCAP 19/26 (Fig. 6B), UTEX 999 (Fig. 6C), and UTEX 1000 (Fig. 6D). In
529 contrast, low salinity stress promoted an increase in total FAs for two strains, *D. tertiolecta*
530 CCAP 19/24 (Fig. 7A) and *D. pseudosalina* CCAP 19/18 (Fig. 7B). The seventh strain, the
531 unique *D. viridis* UTEX ZZ1150, had the highest total FA content at its optimum salinity of
532 60 (Fig. 7C). For example, total FA production by *D. tertiolecta* CCAP 19/9 was
533 significantly higher under increased salinity than at the optimum salinity for population
534 growth, including salinities of 45 ($p = 0.016$), 75 ($p = 0.014$), and 105 ($p < 0.001$) (Table 6).
535 Maximum total FA content (60.6 ± 7.76%; percent total FAs ± 1 standard error [SE])
536 occurred at salinity 105, versus total FA content of only 19.6 ± 1.33% at the optimal salinity
537 (30) for cell production ($p = 0.006$; Table 6, Fig. 6A). In contrast, the total FA content of *D.*
538 *viridis* UTEX ZZ1150 at all salinities was significantly lower than that of control cultures
539 maintained at the optimal salinity for cell production (salinity 60; total FAs 58.2 ± 0.59%; p
540 < 0.0001 to 0.007; Table 6, Fig. 7C).

541 Strain *D. tertiolecta* CCAP 19/24 had significantly lower total FA content at salinity
542 90 than did control cultures for that strain at the optimum salinity (60) for population growth
543 ($p = 0.007$; Table 6). It attained maximal total FA content (35.7 ± 0.86%) under hypo-
544 osmotic salinity stress (salinity 45), whereas the controls had 30.2 ± 1.07% total FA content

545 at salinity 60 (Table 6, Fig. 7A). *D. tertiolecta* UTEX 999 had significantly lower total FA
546 content at salinity 15 than at the control salinity (optimum for cell production, salinity 30; $p = 0.045$) or at higher salinities of 90, 105, and 120 ($p < 0.001$ to 0.009; Table 6). This strain
548 produced comparable total FA content at salinities 30 and 75 (36.4 ± 0.99% to 41.6 ± 6.19%)
549 (Table 6, Fig. 6C).
550
551 3.4.c Growth and long-term salinity stress in mass culture

552 Strains *D. tertiolecta* UTEX 999 and 1000, and *D. viridis* UTEX ZZ1150 were grown
553 separately in mass culture (~150 to 175 L). Each strain had been assessed for population
554 growth at its optimal salinity for cell production in bench-scale experiments (30, for the two
555 *D. tertiolecta* strains; 60 for *D. viridis* UTEX ZZ1150). Strain *D. viridis* was also retested for
556 its response to long-term, decreased salinity. Maximum cell density of *D. tertiolecta* UTEX
557 1000, ~2.5 x 10^6 cells/mL (n = 8), occurred on day 5, whereas highest neutral lipid content
558 (based on visual observations of NR-stained cells under fluorescence microscopy) occurred
559 on day 11 at ~1.0 x 10^6 cells/mL. The large cell size and relatively high cell yield of this
560 strain in association with maximal lipid content made *D. tertiolecta* UTEX 1000 a promising
561 candidate for biofuel production. In addition, the low optimum salinity for its growth (30,
562 versus 60 for *D. viridis* UTEX ZZ1150) would have minimized the cost of culture medium.
563 After several months, however, this strain lost its ability to produce high amounts of lipids,
564 and when an additional culture of the strain was obtained from the UTEX culture collection,
565 it grew slowly and had poor lipid production. Thus, this strain was not considered for further
566 study.

567 The second strain tested in mass culture, *D. tertiolecta* UTEX 999, was unable to
568 sustain rapid growth in mass culture and attained a maximum of only ~2.5 x 10^5 cells/mL at

569 salinity 30, ~ten-fold less than its growth in bench-scale cultures under otherwise-similar
570 conditions. The third strain, *D. viridis* UTEX ZZ1150, grew comparably in bench-scale and
571 mass cultures. At its optimal salinity of 60 for population growth, however, corrosion
572 occurred on overhanging light fixtures and mass culture tubes had to be acid-stripped
573 between experiments due to residue accumulation on the walls. In mass culture, this strain
574 produced two-fold more cells at salinity 60 than at salinity 30 (~4 x 10^6 cells/mL versus ~2 x
575 10^6 cells/mL, respectively), but still had relatively high cell production at the sub-optimal
576 salinity. Thus, *D. viridis* UTEX ZZ1150 was successfully mass-cultured at the sub-optimal as
577 well as the optimal salinity for cell production.

578 3.4.d Short-term salinity stress

579 All three *D. tertiolecta* strains tested in this experimental series (CCAP 19/9, CCAP
580 19/24, and UTEX 999) reached senescence and maximum cell production (~5.0 x 10^5
581 cells/mL) within 7 days of inoculation. The fourth strain assessed in the short-term trials, *D.*
582 *viridis* UTEX ZZ1150, attained maximal cell production (~1.0 to 1.5 x 10^6 cells/mL) more
583 slowly, at 10 days. In general, total FAs increased within 5 min to 1 hr after cultures were
584 exposed to higher salinity (15 or 30 above the optimal in controls; Fig. 8A-C).

585 The three *D. tertiolecta* strains exhibited both immediate and short-term increases in
586 total FA production in response to high salinity stress. At salinity 60, *D. tertiolecta* CCAP
587 19/9 significantly increased its total FA content within 1 hr (34.8 ± 1.30%, total FAs ± 1 SE;
588 $p = 0.015$) in comparison to the control cultures at salinity 30 (22.9 ± 1.80%; Table 7, Fig.
589 8A). At salinity 45, this strain significantly increased its total FA content at 24 hr ($p = 0.036$).
590 Total FA content was comparable to that of controls at all other time intervals.

D. tertiolecta CCAP 19/24 significantly increased in total FA content, relative to the control cultures maintained at salinity 60, at time points of 5 min (36.4 ± 1.69%) and 30 min (33.3 ± 1.76%%) after exposure to salinity 75 ($p = 0.005$ to 0.034) (Table 7, Fig. 8B). At salinity 90, this strain significantly increased in total FA content at 5 min, 30 min, and 24 hr ($p = 0.012$ to 0.050) in comparison to the total FA content of control cultures (24.4 ± 0.1% to 25.9 ± 0.92%; Table 7, Fig. 8B). As with *D. tertiolecta* CCAP 19/9, there was an increase in total FAs at each time point, although not significantly different from the total FA content of controls.

The third *D. tertiolecta* strain, UTEX 999, increased in in total FA content at a salinity of 45 or 60 relative to the control cultures as an immediate response (30 sec to 5 min; Fig. 8C). At salinity 45, its total FAs increased at 30 sec, 5 min and 1 hr ($p = 0.009$ to 0.032) relative to the total FA content of control cultures (Table 7, Fig. 8C). At 30 sec, 5 min, and 30 min after exposure to salinity of 60, *D. tertiolecta* UTEX 999 increased in total FAs when compared to the controls ($p < 0.0001$ to 0.0444). Highest total FAs were measured at 30 sec after exposure to either salinity 45 or salinity 60 (37.8 ± 2.74% and 35.8 ± 2.22%, respectively; Fig. 8C). There was a significant difference in total FAs at 1 hr between the salinity stress treatments (salinity30 to 45, and salinity 30 to 60; $p = 0.017$).

Finally, *D. viridis* UTEX ZZ1150 significantly increased total FA content 30 sec and 5 min after being exposed to salinity 75 ($p < 0.0001$ to 0.0066) and to salinity 90 ($p < 0.0001$ to 0.0014) when compared to its total FA content at salinity 60 (Fig. 8D). There was a significant increase in total FAs at salinity 90 at 30 min and 24 hr after exposure ($p = 0.030$ to 0.041). At 30 sec after exposure, there was a significant difference among treatments (salinity 60 to 75, and salinity 60 to 90; $p = 0.007$). Total FAs increased at each time period

614 regardless of salinity stress when compared to the total FA content of controls (Table 7, Fig.
615 8D).
616
617 3.4.e Total free glycerol

618 Although the total FA content of *D. viridis* UTEX ZZ1150 was comparable at its optimal
619 salinity (60) versus at higher salinities, as described above, the total glycerol content of
620 pelleted *D. viridis* UTEX ZZ1150 increased significantly relative to that of control cultures in
621 response to salinity 90 at 1 hr ($p = 0.03435$) and 2 hr ($p = 0.00665$). Maximum total free
622 glycerol was measured at 24 hr after exposure to salinity 90 (17.8 ± 3.31 mg/g, as total free
623 glycerol ± 1 SE; $p < 0.0001$) (Fig. 9A). In the supernatant from the centrifuged *D. viridis*
624 UTEX ZZ1150 cultures, total free glycerol significantly increased from 30 min up to 24 hr
625 after exposure to salinity 90 ($p < 0.0001$ to 0.0264), with maximal glycerol at 24 hr (0.012
626 mg/mL ± 0.0006) (Fig. 9B).
627
628 3.4.f Time course with real-time quantitative polymerase chain reaction (RT-qPCR)

629 RT-qPCR indicated that *ACTIN* was constitutively expressed by *D. viridis* UTEX
630 ZZ1150 regardless of salinity stress. Stable expression confirmed that *ACTIN* acted as a
631 housekeeping gene throughout the experiment. The $2^{-\Delta\Delta Ct}$ method was used to calculate the
632 relative expression of *GPDH* to the endogenous gene *ACTIN* and to the calibrator sample
633 (Before cDNA). *GPDH* was significantly expressed, when compared to the baseline *ACTIN*
634 expression, at 30 min ($p = 0.0107$), 1 hr ($p = 0.0102$), and 2 hr ($p = 0.0035$) after exposure to
635 hyperosmotic stress. *GPDH* expression continued for 24 hr, but was not statistically
636 significant at that time point (Fig. 10). Expression was evaluated as significant if the n-fold
637 expression was ≥ 2. The corresponding melting curve confirmed a single peak in all

638 dissociation curves, indicating that there was no non-specific amplification of products
639 during the RT- qPCR procedure.
640
641 **3.5 Discussion**
642 Microalgae have high surface area-to-volume ratios, which facilitate rapid responses
643 to changing environmental conditions (Becker, 1994; Rodolfi et al., 2009), and they can
644 quickly alter lipid metabolism in response to environmental stressors (Roessler, 1990) such
645 as salinity stress. This research built from the short-term and long-term experimental
646 approach of Takagi et al. (2006), and examined *rapid*, *short-term*, and *long-term* responses
647 (after Chen and Jiang, 2009) of selected *Dunaliella* strains to environmental stressors. During
648 the short-term experiments, a reversible physiological change was expected, which would
649 have allowed the population to survive during the altered conditions. The length of the long-
650 term salinity stress experiments (2 weeks) would have allowed epigenetic adaptation
651 resulting in the synthesis of salinity-induced proteins and expression of salinity stress genes
652 (Chen and Jiang 2009, Lakeman et al., 2009). Plants have developed a wide range of
653 responses to environmental stressors, such as DNA methylation, chromatin remodeling, and
654 small RNA-based mechanisms that are involved in regulating gene expression especially
655 under abiotic stress (Sahu et al., 2013).
656 As was expected based on previous research with *Dunaliella* (Hanaa et al., 2004 –
657 with *D. salina*, strain number unavailable, from the Botany Department of Texas University;
658 and Takagi et al., 2006 – with *D. tertiolecta* strain ATCC 30929 from the American Type
659 Culture Collection), high salinity stress generally promoted FA production, but very high
660 intraspecific variation was also found. *Dunaliella tertiolecta* strains CCAP 19/9, CCAP
661 19/26, and UTEX 999 increased in total FA content as percent dry weight when exposed to

662 long-term hyperosmotic stress, albeit at different salinity levels (Fig. 6A-C). In contrast, the
663 percent total FA content of *D. viridis* UTEX ZZ1150 *decreased* under high salinity stress,
664 relative to a maximum of nearly 60% (dry weight basis) in the controls (optimal salinity, 60)
665 (Fig. 7C). And, *D. tertiolecta* CCAP 19/24 increased in total FA content under *low* salinity
666 stress (Fig. 7A).

667 In the experiments testing long-term response salinity stress, four of the seven strains
668 increased total FA content under high salinity stress, whereas two strains showed an increase
669 in total FAs under low salinity stress. *D. viridis* UTEX ZZ1150 had the highest amounts of
670 total FAs at its optimal salinity of 60. This small-celled strain grew most rapidly, as expected,
671 in comparison to the other strains tested in this study. It was also unique in two other
672 features: Unlike any of the other 13 strains, it produced neutral lipids during active growth as
673 well as during senescence, and it grew well under continuous light (Mixson, 2013; Mixson et
674 al., submitted). Most microalgae have been found to accumulate most lipids during stationary
675 phase or senescence rather than during active growth (Henderson and Sargent, 1989;
676 Hodgson et al., 1991; Brown et al., 1996; Pond and Harris, 1996; Hu et al., 2008 and
677 references therein; Yoo et al., 2010). Aging or senescence of algal cultures is accompanied
678 by a change in lipid biosynthesis pathways from the chloroplast or other cellular membranes
679 to neutral storage lipids (Qunju et al., 2015). Additionally, most algae that have been tested
680 do not grow well in continuous light (e.g., Carvalho and Malcata, 2004; Lorenz et al., 2005,
681 p.150; Sforza et al., 2012). Typically during the dark cycle, plant cells use FAs that
682 accumulated in the light period for energy (Guckert and Cooksey, 1990; Gardner et al.,
683 2012).

Previous research by Takagi et al. (2006) showed that total lipid content of *D. tertiolecta* ATCC 30929 increased to ~70% whether cultures were grown initially at higher salinity or sustained hyperosmotic stress at the middle or end of the logarithmic growth phase. Concomitantly, however, cell production significantly decreased in response to high salinity stress. Takagi et al. (2006) suggested that this strain of *D. tertiolecta* may be able to recover normal cell production if allowed to acclimate to higher salinity through *gradual* increase of NaCl concentrations. The present study supports that premise: These *Dunaliella* populations grew normally if allowed to acclimate, to a point. Six of the seven strains (exception, *D. pseudosalina* CCAP 19/18) were able to maintain similar growth patterns under increasing salinity, until the salinity was 90 higher than the control (optimum) salinity for the strain. That point marked the limit of tolerance to high salinity stress for the six strains, and cell production significantly declined. In addition, *D. pseudosalina* CCAP 19/18 lacked two FAs that were present in all of the other strains tested. These two FAs, C18:2, cis9,12 and C18:3, cis6,9,12, are unsaturated, meaning that they contain at least one double carbon-carbon bond. The degree of unsaturation is important in cells such as *Dunaliella* because unsaturated FAs influence adaptation to salinity stress (Xu and Beardall 1997, Vanitha et al., 2007). The data suggest that, lacking these unsaturated FAs, *D. pseudosalina* CCAP 19/18 may not have been able to successfully acclimate to higher salinities.

In response to fluctuating salinities, *Dunaliella* generally osmoregulates by producing or releasing glycerol (in hyperosmotic or hypo-osmotic conditions, respectively) as a mechanism to return the cells to their original volume (Chitlaru and Pick, 1991; Ginzburg et al., 1995; Ben-Amotz et al., 2009; Chen and Jiang, 2009). Based on research with various *Dunaliella* strains, cell size and shape are known to decrease immediately after exposure to

hyperosmotic stress (Avron and Ben-Amotz, 1992; Ben-Amotz et al., 2009; Chen and Jiang, 2009). As shown in this study, total FA content significantly increased in tested strains within sec to hr after exposure to high salinity. These responses were strain-specific. Lipid composition was similar, however, throughout the short-term study. Like various other algae, *Dunaliella* lacks a polysaccharide cell wall; instead, the outer covering is a thin membrane covered by a fibrous extracellular matrix (Graham et al., 2016). The lack of a rigid cell wall facilitates rapid changes in volume and shape in response to changes in salinity (Chitlaru and Pick, 1991; Ben-Amotz et al., 2009; Chen and Jiang, 2009). Cells typically maintain constant volume after the immediate exposure, independent of external salinity (Avron, 1986; Azachi et al., 2002). The glycerol experiment narrowed the window of time during which glycerol significantly increases in response to hyper-osmotic shock (sudden salinity increase from 60 to 90) for up to ~1 hr after exposure, earlier than proposed by Chen and Jiang (2009). If confronted by hyper-osmotic stress, cells increase their total FA content as an immediate or short-term response. This increase may occur because the cells incorporate a proportion of the available glycerol as TAGs, thus explaining the low amounts of total free glycerol. As a long-term response (i.e. 24 hr), intracellular glycerol significantly increased (Fig. 9). In addition, the cells likely began dividing at or after 24 hr and the available glycerol would then have been metabolized into other products such as starch, sugars, or pyruvate required for active growth. Future experiments will be needed to assess when cell division occurs after cells are exposed to salinity stress.

Glycerol and glycerol biosynthesis genes have been extensively studied in *Dunaliella* because of their importance in osmoregulation. The amount excreted or produced under hypo-osmotic and hyperosmotic conditions depends mostly on the concentration of NaCl in

730 the surrounding medium (Borowitzka and Brown, 1974; Chitlaru and Pick, 1991; Ginzburg
731 et al., 1995; Ben-Amotz et al., 2009), and can be as high as > 6 M in media with salinity
732 greater than 230 (Oren, 2005). In agreement with other studies on *Dunaliella* (Ben-Amotz,
733 1973; Belmans and Van Laere, 1987; Goyal, 2007), in this study glycerol began increasing
734 within 30 min of exposure to hyperosmotic stress and continued to accumulate up to 24 hr. A
735 steady-state condition was not observed; similarly, work by other researchers has shown that
736 glycerol may continue to be produced for 1 to 1.5 hr when the cells attain an internal glycerol
737 concentration similar to that of fully adapted cells (Ben-Amotz and Avron, 1973; Belmans
738 and Van Laere, 1987; Goyal, 2007). In this study at 24 hr after exposure to high salinity
739 stress, the internal glycerol concentration had increased to double that of control populations.
740 Similar findings were reported by He et al. (2007).
741 Many previous studies investigating glycerol accumulation have examined only one
742 time point at the end of the experiment. The time course experiment in this research was
743 similar to that of Belmans and Van Laere (1987); both studies analyzed intracellular glycerol
744 accumulation within sec to min up to 2 hr after hyperosmotic shock, but this study
745 additionally extended to 24 hr. Both Belmans and Van Laere (1987) and this work detected
746 an increase in glycerol immediately after hyperosmotic shock, and a steady-state condition at
747 2 hr. In addition, it was established that glycerol continued to accumulate after 24 hr before
748 the cells fully adapted, well beyond the apparently temporary steady-state condition detected
749 at 2 hr. Had sampling ended at 2 hr, the accumulation of glycerol mistakenly would have
750 appeared to reach steady-state, when in reality the population required considerably longer to
751 fully adapt to hyperosmotic stress.

In the pathway of glycerol biosynthesis, *GPDH* is an important enzyme converting DHAP to G-3-P, which is then converted to the end product glycerol by the enzyme *GPI* (Haus and Wegmann, 1984; Chen and Jiang, 2009). This enzyme is also referred to as *DHAP reductase* because at physiological conditions (i.e. pH and substrate) it is inactive as a dehydrogenase (Gee et al., 1988). The RT-qPCR data from this study showed that the relative expression of *GPDH* increased 30 min, 1 hr, and 2 hr after high salinity stress. These data supported the findings for total free glycerol (Fig. 10). At 24 hr, maximum total glycerol was found; however, this timing did not correlate with a significant expression of *GPDH*. It is likely that because glycerol was produced in large quantities, *GPDH* was no longer up-regulated.

This gene expression experiment was only completed for the strain *D. viridis* UTEX ZZ1150. In further research, gene expression could be investigated in other strains to assess whether *GPDH* expression correlates with glycerol production and/or total FA production. Other researchers have conducted similar measurements on *Dunaliella* (identified by the culture collection as *D. salina* UTEX 200) after 2 hr of high salinity stress (e.g. Chen et al., 2011). This study with *D. viridis* UTEX ZZ1150 used shorter time periods (sec to min) in order to capture more rapid changes, and showed that *GPDH* begins to be expressed (at activities at least 2-fold higher than *GPDH* activities of unstressed control populations) within 30 min after exposure to high salinity stress, continuing to 2 hr. In general, when total free glycerol was low, the four strains that were tested increased in total FAs, but there were considerable differences among the strains in their response. This work builds from Belmans and Van Laere (1987), who showed increased activity of *GPDH* due to the cellular increase of G-3-P content during the period of rapid glycerol synthesis. Triose phosphates and

775 fructose-1,6-bisphosphate remained constant during the same period, suggesting *GPDH*
776 activity (Belmans and Van Laere, 1987). These RT-qPCR data provide evidence supporting
777 the premise that *GPDH* is up-regulated in response to hyperosmotic stress. As indicated
778 above, glycerol is involved in many metabolic pathways. Future work should include
779 quantification of total starch or sugars to further validate the glycerol results.
780
781 **3.6 Conclusion**

782 Among 14 strains within four *Dunaliella* species that were screened for cell
783 production and lipid content, seven strains were selected for tests of enhanced lipid
784 production under both low and high salinity stress in long-term (≥ 24 hr) experiments, and
785 four of these strains additionally were tested for their short-term response (sec or min to less
786 than 24 hr). Under long-term high salinity stress, *Dunaliella tertiolecta* CCAP 19/9 produced
787 the highest proportion of total FAs per unit cell mass (up to 65% by dry weight) relative to
788 controls (~10-25% total FAs). In the short-term tests, FA production significantly increased
789 within sec to hr of exposure to high salinity stress. The most promising strain, *D. viridis*
790 UTEX ZZ1150, was shown to produce glycerol within 30 min to 24 hr. Total free glycerol
791 was low inside the cell when total FAs were increasing as an immediate or short-term
792 response to hyperosmotic stress, suggesting that the cells are incorporating a portion of the
793 free glycerol as TAGs. Strain *D. viridis* UTEX ZZ1150 was also successfully mass-cultured
794 (~150 to 175 L) with high cell production in salinities of 60 or 30.
795
796 **Acknowledgments**

797 This study was funded by the National Science Foundation EFRI program (grant EFRI-
798 093772) and the North Carolina General Assembly. We thank the CAAE staff for their many
799 helpful efforts, such as Robert Reed and Eric Morris for their extensive work in setting up the

mass culture facilities, and Elle Allen for assistance in culturing and microscopy. Brian McCann, Abhi Bhargava, Andrew Campos, and Wilson Wang assisted in mass culture and work with the gas chromatograph/flame ionization detector, and Jake Dums assisted in photography. We also thank two anonymous reviewers for their constructive comments.

3.7 References

Araujo, G.S., Matos, L.J.B.L., Gonçalves, L.R.B., Fernandez, F.A.N., Farias, W.R.L., 2011. Bioprospecting for oil producing microalgal strains: Evaluation of oil and biomass production for ten microalgal strains. Bioresour. Technol. 102, 5248-5250.

Arias-Forero, D., Hayashida, G., Aranda, M., Araya, S., Portilla, T., Garcías, A., Díaz-Palma, P., 2013. Protocol for maximizing the triglycerides-enriched lipids production from *Dunaliella salina* SA32007 biomass, isolated from the Salar de Atacama (northern Chile). Advances in Bioscience and Biotechnology 4, 830-839.

Assunção, P., Jaén-Molina, R., Caujapé-Castells, J., de la Jara, A., Carmona, L., Freijanes, K., Mendoza, H., 2012. Molecular taxonomy of *Dunaliella* (Chlorophyceae), with a special focus on *D. salina*: ITS2 sequences revisited with an extensive geographical sampling. Aquatic Biosystems 8, 2.

Avron, M., 1986. The osmotic components of halotolerant algae. Trends Biochem. Sci. 11, 5-6.

Avron, M., Ben-Amotz, A. (Eds.), 1992. *Dunaliella*: Physiology, Biochemistry, and Biotechnology. CRC Press, Boca Raton (Florida). 256 pp.

Azachi, M., Sadka, A., Fisher, M., Goldshlag, P., Gokhman, I., Zamir, A., 2002. Salt induction of fatty acid elongase and membrane lipid modifications in the extreme halotolerant alga *Dunaliella salina*. Plant Physiol. 129, 1320-1329.

824 Becker, E.W., 1994. Microalgae: Biotechnology and Microbiology. Cambridge University
825 Press, New York.
826 Belmans, D., Van Laere, A., 1987. Glycerol cycle enzymes and intermediates during
827 adaptation of *Dunaliella tertiolecta* cells to hyperosmotic stress. Plant Cell Environ. 10,
828 185-190.
829 Ben-Amotz, A., Avron, M., 1973. The role of glycerol in the osmotic regulation of the
830 halophilic alga *Dunaliella parva*. Plant Physiol. 51, 875-878.
831 Ben-Amotz, A., and Avron, M., 1989. The Biotechnology of Mass Culturing *Dunaliella* for
832 Products of Commercial Interest. *In* Creeswell, R.C., Rees, T.A.V, and Shah, N. [Eds.].
833 Algal and Cyanobacterial Biotechnology, Longman Scientific and Technical Press, New
834 York, pp. 90-114.
835 Ben-Amotz, A., Polle, J.E.W., Rao, D.V.S. (Eds.), 2009. The Alga *Dunaliella*: Biodiversity,
836 Physiology, Genomics and Biotechnology. Science Publishers, Enfield (New
837 Hampshire).
838 Bligh, E.G., Dyer W.J., 1959. A rapid method of total lipid extraction and purification. Can.
839 J. Biochem Physiol 37, 911-917.
840 Borowitzka, L.J., Brown, A.D., 1974. The salt relations of marine and halophilic species of
841 the unicellular green alga, *Dunaliella*. Arch. Microbiol. 96, 37-52.
842 Borowitzka, M.A., Siva, C.J., 2007. The taxonomy of the genus *Dunaliella* (Chlorophyta,
843 Dunaliellales) with emphasis on the marine and halophilic species. J. Appl. Phycol. 19,
844 567-590.

845 Brown, M.R., Dunstan, G.A., Norwood, S.J., Miller, K.A., 1996. Effects of harvest stage and
846 light on the biochemical composition of the diatom *Thalassiosira pseudonana*. J. Phycol.
847 32, 64-73.
848 Bruttel, P., Schlink, R., 2003. Water determination by Karl Fischer titration. Met Mono 1, 1-
849 80.
850 Carvalho, A., Malcata, F.X., 2004. Optimization of ω-3 fatty acid production by microalgae:
851 crossover effects of CO_2 and light intensity under batch and continuous cultivation
852 modes. Mar. Biotechnol. 7, 381-388.
853 Chen, H., Jiang, J.G., 2009. Osmotic responses of *Dunaliella* to the changes of salinity. J.
854 Cell Physiol. 219, 251-258.
855 Chen, H., Lao, Y.-M., Jiang, J.-G., 2011. Effects of salinities on the gene expression of a
856 (NAD^+)-dependent glycerol-3-phosphate dehydrogenase in *Dunaliella salina*. Sci. Total
857 Environ. 409, 1291-1297.
858 Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K.Y.S., Salley, S.O., 2009. Effect of
859 nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*.
860 Bioresour. Technol. 102, 1649-1655.
861 Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126-
862 131.
863 Chitlaru, E., Pick, U., 1991. Regulation of glycerol synthesis in response to osmotic changes
864 in *Dunaliella*. Plant Physiol. 96, 50-60.
865 Cooksey, K.E., Guckert, J.B., Williams, S.W., Callis, P.R., 1987. Fluorometric determination
866 of the neutral lipid content of microalgal cells using Nile Red. J. Microbiol. Methods 6,
867 333-345.

868 Coons, J.E., D.M. Kalb, D.M., Dale, T., Marrone, B.L., 2014. Getting to low-cost algal
869 biofuels: A monograph on conventional and cutting-edge harvesting and extraction
870 technologies. Algal Research 6, 250-270.
871 Edwards, K., Logan, J., Saunders, N. (Eds.), 2004. Real-Time PCR: An Essential Guide.
872 Horizon Bioscience, Norfolk (United Kingdom).
873 Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and
874 purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.
875 Føyn, B. 1934. Lebenszyklus, Cytologie und Sexualiät der Chlorophycee *Cladophora*
876 *suhriana* Kützing. Arch. Protistenk. 83, 1-56.
877 García-González, M., Moreno, J., Cañavate, J.P., Anguis, V., Prieto, A., Manzano, C.,
878 Florencio, F.J., Guerrero, M.G., 2003. Conditions for open-air outdoor culture of
879 *Dunaliella salina* in southern Spain. J. Appl. Phycol. 15, 177-184.
880 Gardner, R.D., Cooksey, K.E., Mus, F., Macur, R., Moll, K., Eustance, E., Carlson, R.P.,
881 Gerlach, R., Fields, M.W., Peyton, B.M., 2012. Use of sodium bicarbonate to stimulate
882 triacylglycerol accumulation in the chlorophyte *Scenedesmus* sp. and the diatom
883 *Phaeodactylum tricornutum*. J. Appl. Phycol. 24, 1311-1320.
884 Gee, R., Goyal, A., Gerber, D., Byerrum, R.U., Tolbert, N.E., 1988. Isolation of
885 dihydroxyacetone phosphate reductase from *Dunaliella* chloroplasts and comparison with
886 isozymes from spinach leaves. Plant Physiol. 88, 896-903.
887 Ginzburg, M., 1987. *Dunaliella*: a green alga adapted to salt. Adv. Bot. Res. 14, 93-183.
888 Ginzburg, B.Z., Ginzburg, M., Cohen, M., 1995. A salt-sensitive *Dunaliella* mutant I.
889 Growth and osmoregulatory responses to increases in salt concentration. J. Exp. Bot. 46,
890 1505-1513.

891 Goff, L.J., Moon, D.A., Coleman, A.W. 1994. Molecular delineation of species and species
892 relationships in the red algal agarophytes *Gracilariopsis* and *Gracilaria* (Gracilariales).
893 J. Phycol. 30, 521-537.
894 Gómez, P., González, M., 2004. Genetic variation among seven strains of *Dunaliella salina*
895 (Chlorophyta) with industrial potential, based on RAPD banding patterns and on nuclear
896 ITS rDNA sequences. Aquaculture 233, 149-162.
897 González, M., Gómez, P. & Montoya, R. 1998. Comparison of PCR-RFLP analysis of the
898 ITS region with morphological criteria of various strains of *Dunaliella*. J. Appl. Phycol.
899 10, 573-580.
900 Goyal, A., 2007. Osmoregulation in *Dunaliella*, part II: photosynthesis and starch contribute
901 carbon for glycerol synthesis during a salt stress in *Dunaliella tertiolecta.* Plant Physiol.
902 Biochem. 45, 705-710.
903 Graham, L.E., Graham, L.W. Wilcox, L.W., Cook, M.E., 2016. Algae, 3rd edition. LJLM Press,
904 LLC.
905 Griffiths, M.J., van Hille, R.P., Harrison, S.T.L., 2010. Selection of direct transesterification
906 as the preferred method for assay of fatty acid content of microalgae. Lipids 45, 1053-
907 1060.
908 Guckert, J.B., Cooksey, K.E., 1990. Triglyceride accumulation and fatty acid profile changes
909 in *Chlorella* (Chlorophyta) during high pH-induced cell cycle inhibition. J. Phycol. 26,
910 72-79.
911 Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith,
912 W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New
913 York, pp. 29-60.

914 Guillard, R.R.L., 2005. Purification methods for microalgae. In: Andersen, R.A. (Ed.), Algal
915 Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts), pp. 117-132.
916 Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte.
917 Phycologia 32, 234-236.
918 Hanaa, H., El-Baky, A., El-Baz, F.K., El-Baroty, G.S., 2004. Production of lipids rich in
919 omega 3 fatty acids from the halotolerant alga *Dunaliella salina*. Biotechnology 3, 102-
920 108.
921 Haus, M., Wegmann, K., 1984. Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) from
922 *Dunaliella tertiolecta.* I. Purification and kinetic properties. Physiol. Plant. 60, 283-288.
923 He, Q., Qiao, D., Bai, L., Zhang, Q., Yang, W., Li, Q., Cao, Y., 2007. Cloning and
924 characterization of a plastidic glycerol 3-phosphate dehydrogenase cDNA from
925 *Dunaliella salina*. J. Plant Physiol. 164, 214-220.
926 Henderson, R.J., Sargent, J.R., 1989. Lipid composition and biosynthesis in ageing cultures
927 of the marine cryptomonad, *Chroomonas salina*. Phytochemistry 28, 1355-1361.
928 Hillebrand, H., Dürselen, C.-D., Kirschtel, D., Pollingher, U., Zohary, T., 1999. Biovolume
929 calculation for pelagic and benthic microalgae. J. Phycol. 35, 403-424.
930 Hodgson, P.A., Henderson, R.J., Sargent, J.R., Leftley, J.W., 1991. Patterns of variation in
931 the lipid class and fatty acid composition of *Nannochloropsis oculata*
932 (Eustigmatophyceae) during batch culture. I. The growth cycle. J. Appl. Phycol. 3, 169–
933 181.
934 Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A.,
935 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and
936 advances. Plant J. 54, 621-639.

937 Kawachi, M., Noël, M.-H., 2007. Sterilization and sterile technique. In: Andersen, R.A.
938 (Ed.), Algal Culturing Techniques. Elsevier Academic Press, Burlington (Massachusetts),
939 pp. 65-81.
940 Keller, A., Förster, F., Müller, T., Dandekar, T., Shultz, J., Wolf, M., 2010. Including RNA
941 secondary structures improves accuracy and robustness in reconstruction of phylogenetic
942 trees. Biology Direct 5, 4.
943 Klatte, M., Bauer, P., 2009. Accurate real-time reverse transcription quantitative PCR. Plant
944 Sig. Transduct. 479, 61-77.
945 Lakeman, M.B., von Dassow, P., Cattolico, R.A. 2009. The strain concept in phytoplankton
946 ecology. Harmful Algae. 8, 746-758.
947 Lee, S.J., Yoon, B.-D., Oh, H.-M., 1998. Rapid method for the determination of lipid from the
948 green alga *Botryococcus braunii*. Biotechnology Techniques 12, 553-556.
949 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
950 quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402-408.
951 Lorenz, M., Friedl, T., Day, J.G., 2005. Perpetual Maintenance of Actively Metabolizing
952 Microalgal Cultures. In: Andersen, R.A. (Ed.), Algal Culturing Techniques. Elsevier
953 Academic Press, Burlington (Massachusetts). pp. 145-156.
954 Massjuk, N.P., 1973. Morphology, taxonomy, ecology, and geographic distribution of the
955 genus *Dunaliella* Teod and prospects for its potential utilization. Naukova Dumka, 242
956 pp.
957 Mata, T.M., A.A. Martins, and N.S. Caetano. 2010. Microalgae for biodiesel production and
958 other applications: A review. Renewable and Sustainable Energy Reviews 14, 217-232.

959 Miner, C.S., Dalton, N.N. (eds.), 1953. Glycerol. Reinhold Publishing Corporation, New
960 York.
961 Mixson, S.M., 2013. *Dunaliella* spp. under environmental stress: Enhancing lipid production
962 and optimizing harvest. Ph.D. thesis, Department of Plant Biology, North Carolina State
963 University, Raleigh.
964 Mixson, S.M., Burkholder, submitted. Environmental stressors and lipid production in
965 Dunaliella spp. II. Nutrients, pH, and light under optimal or low salinity. J. Exp. Mar.
966 Biol. Ecol.
967 Mixson, S.M., Stikeleather, L.F., Simmons, O.D. III, Wilson, C.W., Burkholder, J.M., 2014.
968 pH-induced flocculation, indirect electrocoagulation, and hollow fiber filtration
969 techniques for harvesting the saltwater microalga *Dunaliella*. J. Appl. Phycol., DOI
970 10.1007/s10811-013-0232-z.
971 Müller, T., Philippi, N., Dandekar, T., Shultz, J., Wolf, M., 2007. Distinguishing species.
972 RNA 13, 1469-1472.
973 Mus, F., Toussaint, J., Cooksey, K.E., Fields, M.W., Gerlach, R., Peyton, B.M., Carlson,
974 R.P., 2013. Physiological and molecular analysis of carbon source supplementation and
975 pH stress-induced lipid accumulation in the marine diatom *Phaeodactylum tricornutum*.
976 Appl. Microbiol. Biotechnol. 97, 3625-3642.
977 Olmos-Soto, J., Paniagua-Michel, J., Contreras, R., Trujillo, L., 2002. Molecular
978 identification of β-carotene hyper-producing strains of *Dunaliella* from saline
979 environments using species-specific oligonucleotides. Biotechnology Letters 24, 365-
980 369.
981 Oren, A., 2005. A hundred years of *Dunaliella* research: 1905-2005. Aquat. Biosyst. 1, 1-14.

982 Patee, H.E., Isleib, T.G., Giesbrecht, F.G, McFeeters, R.F., 2000. Investigations into
983 genotypic variations of peanut carbohydrates. J. Agric. Food Chem. 48, 750-756.
984 Pond, D.W., Harris, R.P., 1996. The lipid composition of the coccolithophore *Emiliania*
985 *huxleyi* and its possible ecophysiological significance. J. Mar. Biol. Assoc. UK 76, 579-
986 594.
987 Qunju, H., Wenzhou, X., Shikun, D., Tao, L., Fangfang, Y., Qikun, J., Guanghua, W.,
988 Hualian, W., 2015. The influence of cultivation period on growth and biodiesel properties
989 of microalga *Nannochloropsis gaditana* 1049. Bioresour. Technol. 192, 157-164.
990 Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R.,
991 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass
992 cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100-112.
993 Roessler, P.G., 1990. Environmental control of glycolipid metabolism in microalgae:
994 commercial implications and future research directions. J. Phycol. 26, 393-399.
995 Rozen, S. Skaletsky, H.J., 1998. *Primer3*. Code available at http://www-
996 genome.wi.mit.edu/genome_software/other/primer3.html.
997 Sahu, P.P., Pandey, G., Sharma, N., Puranik, S., Muthamilarasan, M., Prasad, M. 2013.
998 Epigenetic mechanisms of plant stress responses and adaptation. Plant Cell. Rep. 32,
999 1151-1159.
1000 SAS Institute, Inc., 2009. SAS/STAT Guide for Personal Computers, version 9.2. SAS
1001 Institute, Inc., Cary (NC).
1002 Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H., Posten, C.,
1003 Kruse, O., Hankamer, B., 2008. Second generation biofuels: high-efficiency microalgae
1004 for biodiesel production. Bioeng. Res. 1, 20-43.

1005 Schlagermann, P., Göttlicher, G., Dillschneider, R., Rosello-Sastre, R., Posten, C., 2012.
1006 Composition of algal oil and its potential as biofuel. Journal of Combustion, 14 pp.;
1007 doi:10.1155/2012/285185.
1008 Sforza, E., Simionato, D., Giacometti, G.M., Bertucco, A., Morosinotto, T., 2012. Adjusted light
1009 and dark cycles can optimize photosynthetic efficiency in algae growing in photobioreactors.
1010 PLoS ONE 7:e38975.
1011 Shariati, M., Hadi, M.R. 2011. Microalgal biotechnology and bioenergy in *Dunaliella*. Prog.
1012 Molec. Environ. Bioeng. 22, 483-506.
1013 Sparkman, O.D., Penton, Z.E., Kitson, F.G., 2011. Gas Chromatography and Mass
1014 Spectrometry: A Practical Guide, 2nd edition. Elsevier Inc., Burlington (Massachusetts).
1015 Srirangan, S., Sauer, M.-L., Howard, B., Dvora, M., Dums, J., Backman, P., Sederoff, H., 2015.
1016 Interaction of temperature and photoperiod increases growth and oil content in the marine
1017 microalgae *Dunaliella viridis*. PLoS One 10(5): e0127562. doi:
1018 10.1371/journal.pone.0127562.
1019 Stewart, C.N. & Via, L.E. 1993. A rapid CTAB DNA isolation technique useful for RAPD
1020 fingerprinting and other PCR applications. BioTechniques 14: 748-749.
1021 Taiz, L., Zeiger, E., 2006. Plant Physiology, 4th edition. Sinauer Associates, Inc., Sunderland
1022 (Massachusetts).
1023 Takagi, M., Karseno, Yoshida, T., 2006. Effect of salt concentration on intracellular
1024 accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J.
1025 Biosci. Bioeng. 101, 223-226.

1026 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5:
1027 molecular evolutionary genetics analysis using maximum likelihood, evolutionary
1028 distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731-2739.
1029 Tompkins, J., DeVille, M.M., Day, J.G., Turner, M.F., 1995. Culture Collection of Algae and
1030 Protozoa. Catalog of Strains. Ambleside (United Kingdom).
1031 Valenzuela, J., Carlson, R.P., Gerlach, R., Cooksey, K.E., Peyton, B.M., Bothner, B., Fields,
1032 M.W., 2013. Nutrient re-supplementation arrests bio-oil accumulation in Phaeodactylum
1033 tricornutum. Appl. Microbiol. Biotechnol. 97, 7049-7059.
1034 Vanitha, A., Narayan, S., Murthy, K.N.C., Ravishankar, G.A. 2007. Comparative study of
1035 lipid composition of two halotolerant alga, *Dunaliella bardawil* and *Dunaliella salina*.
1036 Int. J. Food Sci. Nutr. 58, 373-382.
1037 Vollenweider, R.A., Talling, J.F., Westlake, D.F. (Eds.), 1974. A Manual on Methods for
1038 Measuring Primary Production in Aquatic Environments, 2nd edition. Blackwell
1039 Scientific, Oxford, United Kingdom.
1040 Walker, T.L., Black, D., Becker, D.K., Dale, J.L., Collet, C., 2005. Isolation and
1041 characterisation of components of the *Dunaliella tertiolecta* chloroplast genome. J. Appl.
1042 Phycol. 17: 495-508.
1043 Wang, W.-C., Allen, E., Campos, A.A., Cade, R.K., Dean, L., Dvora, M., Immer, J.G.,
1044 Mixson, S., Srirangan, S., Sauer, M.-L., Schreck, S., Sun, K., Thapaliya, N., Wilson, C.,
1045 Burkholder, J., Grunden, A.M., Lamb, H.H., Sederoff, H., Stikeleather, L.F., Roberts,
1046 W.L., 2013. ASI: *Dunaliella* marine microalgae to drop-in replacement liquid
1047 transportation fuel. Environmental Progress (November): DOI:10.1002/ep.11855.

1048 Wetzel, R.G., Likens, G.E., 2010. Limnological Analyses, 3rd edition. W.B. Saunders, New
1049 York.
1050 Xu, X.-Q., Beardall, J., 1997. Effect of salinity on fatty acid composition of a green
1051 microalga from an Antarctic hypersaline lake. Phytochemistry 45, 655-658.
1052 Yao, S., Lu, J., Sárossy, Baggesen, C., Brandt, A., An, Y., 2016. Neutral lipid production in
1053 *Dunaliella salina* during osmotic stress and adaptation. J. Appl. Phycol., DOI
1054 10.1007/s10811-016-0794-7.
1055 Yoo, C., Jun, S.-Y., Lee, J.-Y., Ahn, C.-Y., Oh, H.-M., 2010. Selection of microalgae for
1056 lipid production under high levels carbon dioxide. Bioresour. Technol. 101: S/71-S/74.
1057 Zhu, C.J., Lee, Y.K., 1997. Determination of biomass dry weight of marine microalgae. J.
1058 Appl. Phycol 9, 189-194**.**
1059

Figure Legends

Fig. 1. Simplified schematic of glycerol metabolism in *Dunaliella*. Reversible reactions are indicated by the double arrow and important enzymes are indicated by italicized abbreviations (*G6P-I*, glucose-6-phosphate isomerase; *PF6P-P*, pyrophosphate fructose-6-phosphate phosphotransferase; *F1,6-A*, fructose 1,6-bisphosphate aldolase; *GPDH*, glycerol phosphate dehydrogenase; *GPI*, glycerol phosphate isomerase; and the intermediate products of *DHA*, dihydroxyacetone; *DHAP*, dihydroxyacetone phosphate; *PEP*, phosphoenolpyruvate).

Fig. 2. Light micrographs of the 14 strains of *Dunaliella* spp. used in the salinity stress experiments, showing a cell of average size for each strain (600x, scale bar = 5µm), and using the species identifications indicated by the ITS analysis after strain purification: (a) *Dunaliella parva* CCAP 19/9; (b) *D. parva* CCAP 19/26; (c) *Dunaliella primolecta* UTEX 1000; (d) *Dunaliella tertiolecta* CCAP 19/24; (e) *D. tertiolecta* NCMA 364; (f) *D. tertiolecta* NCMA 1320; (g) *D. tertiolecta* UTEX 999; (h) *D. parva* UTEX 1983; (i) *Dunaliella salina* CCAP 19/18; (j) *D. salina* UTEX 200; (k) *D. parva* CCAP 19/10; (l) *D. salina* CCAP 19/3; (m) *D. salina* UTEX 1644; and (n) *Dunaliella bardawil* ATCC 30861. Note that all strains were grown in identical culture conditions and all were photographed at the same time of day to minimize potential cell-cycle size differences.

Fig. 3. Phylogenetic tree for the 14 *Dunaliella* strains (using the names indicated by the culture collections), derived from the ITS2 region of the ITS rDNA sequences with supporting bootstrap values (100 replicates). A ~227 bp product was sequenced for the ITS2 region for each strain and the ITS2 alignment displayed many bp differences, indicative of significant interspecific variation.

Fig. 4. Confocal micrograph of *D. tertiolecta* NCMA 364 – epifluorescence of a cell stained with Nile Red, showing neutral lipids (white arrows; 488 nm excitation, 545-655 nm emission, 400x). Micrograph: E Johannes in Mixson (2013); also in Wang et al. (2013).

Fig. 5. Population growth curves as cell production for the 7 strains of *Dunaliella* selected for long-term salinity stress (see text for culture conditions), including (A) *D. pseudosalina* CCAP 19/18, (B) *D. tertiolecta* strains CCAP 19/9, CCAP 19/24, CCAP 19/26, UTEX 999, UTEX 1000; and (C) *D. salina* (formerly UTEX *D. viridis*, contaminated). Data are given as means ± 1 SE (n = 3).

Fig. 6. Total FAs (as percent dry weight) for four strains of *D. tertiolecta* under long-term salinity stress, including (A) strain CCAP 19/9, (B) strain CCAP 19/26, (C) UTEX 999; and (D) UTEX 1000. These strains had an optimal salinity of 30. Significant differences from the control are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

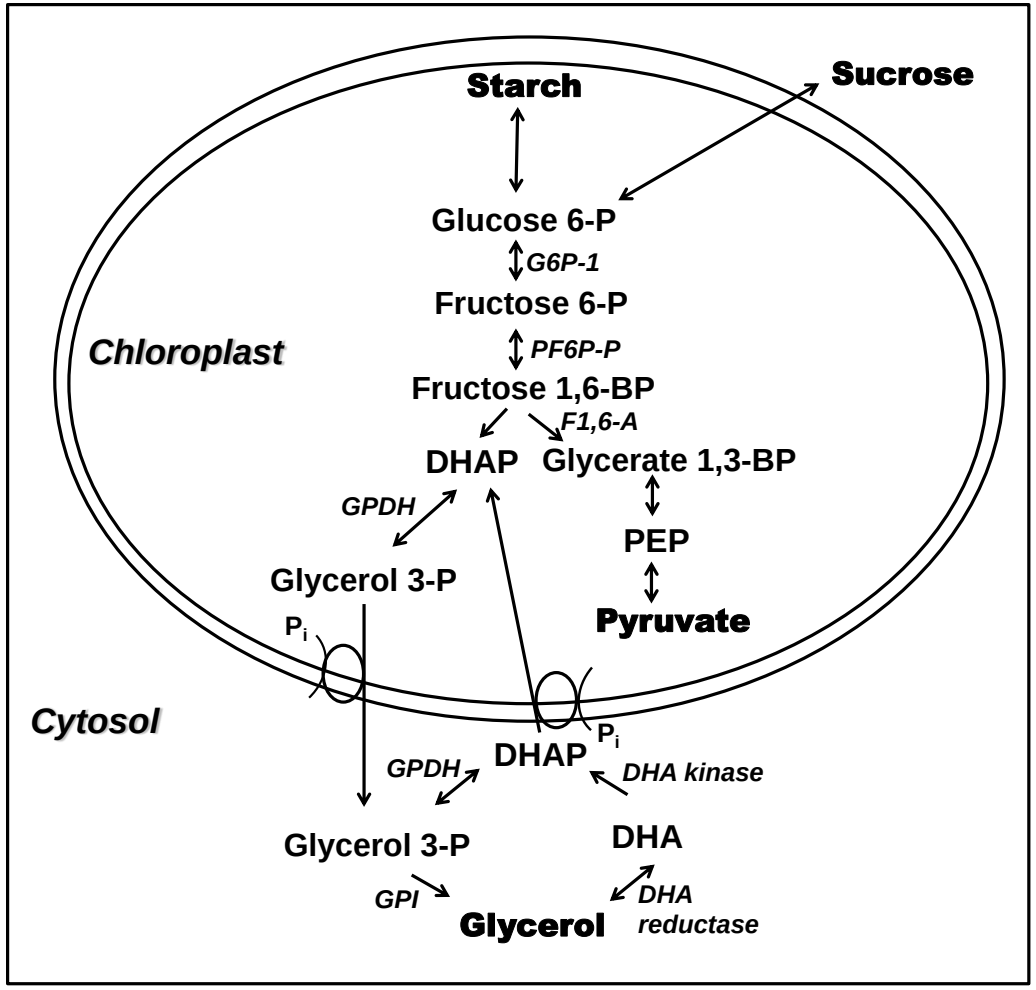
Fig. 7. Total FAs (as percent dry wt) under long-term salinity stress for: (A) *D. tertiolecta* CCAP 19/24; (B) *D. pseudosalina* CCAP 19/18; and (C) *D. viridis* UTEX ZZ1150. Note differences in scale among the three figures. Significant differences from the control (optimum salinity 60 for all three of these strains) are indicated by an asterisk ($p \leq 0.05$); data are given as means $\pm$ 1 SE.

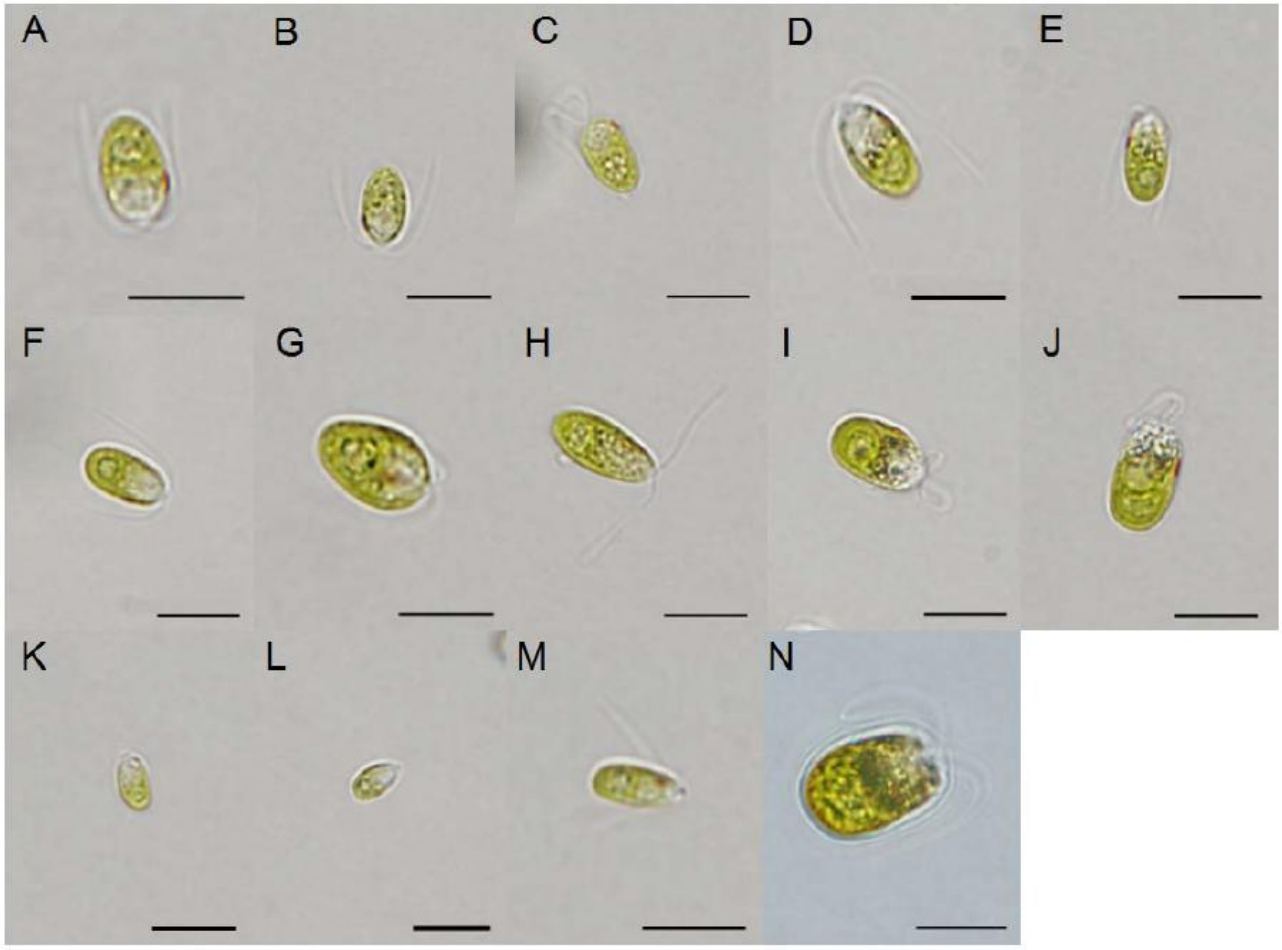
Fig. 8. Total FAs (as percent dry wt) under short-term high salinity stress for: (A) *D. tertiolecta* CCAP 19/9 when control salinity 30 was increased to 45 or 60; (B) *D. tertiolecta* CCAP 19/24 when control salinity 60 was increased to 75 or 90; (C) *D. tertiolecta* UTEX 999 when control salinity 30 was increased to 45 or 60; and (D) *D. viridis* UTEX ZZ1150,

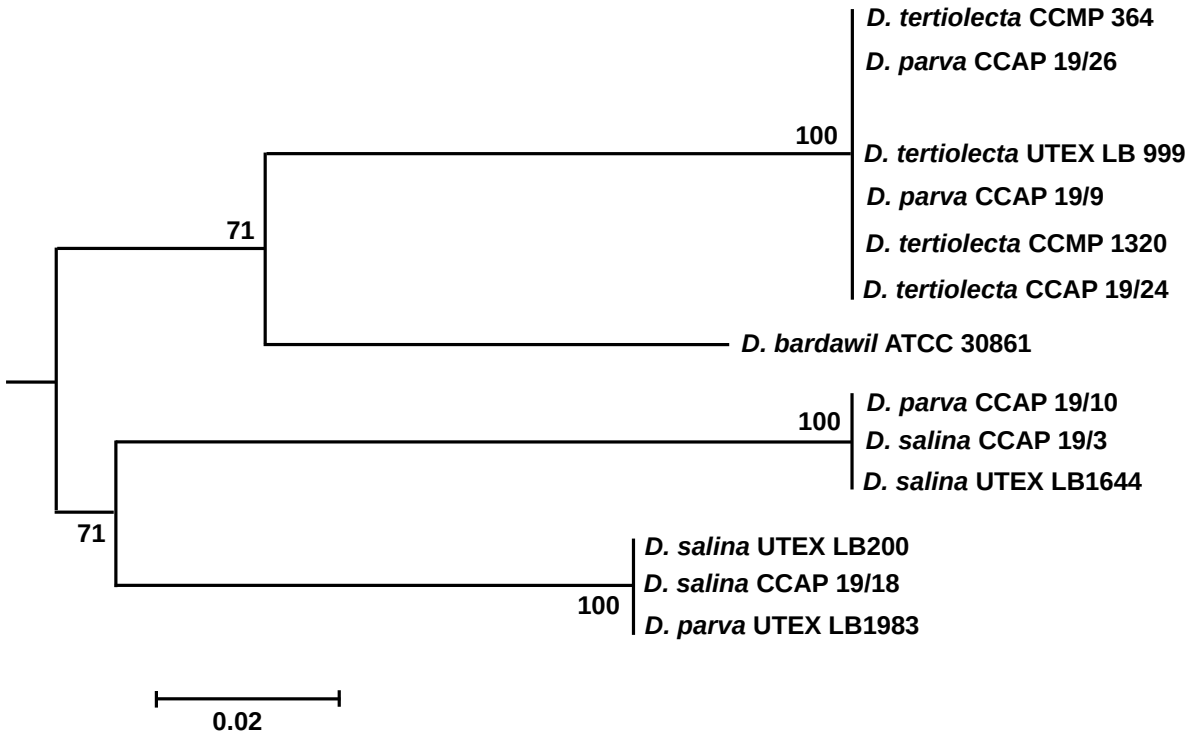
1111 when control salinity 60 was increased to 75 or 90. Significant differences from the control
1112 are indicated by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

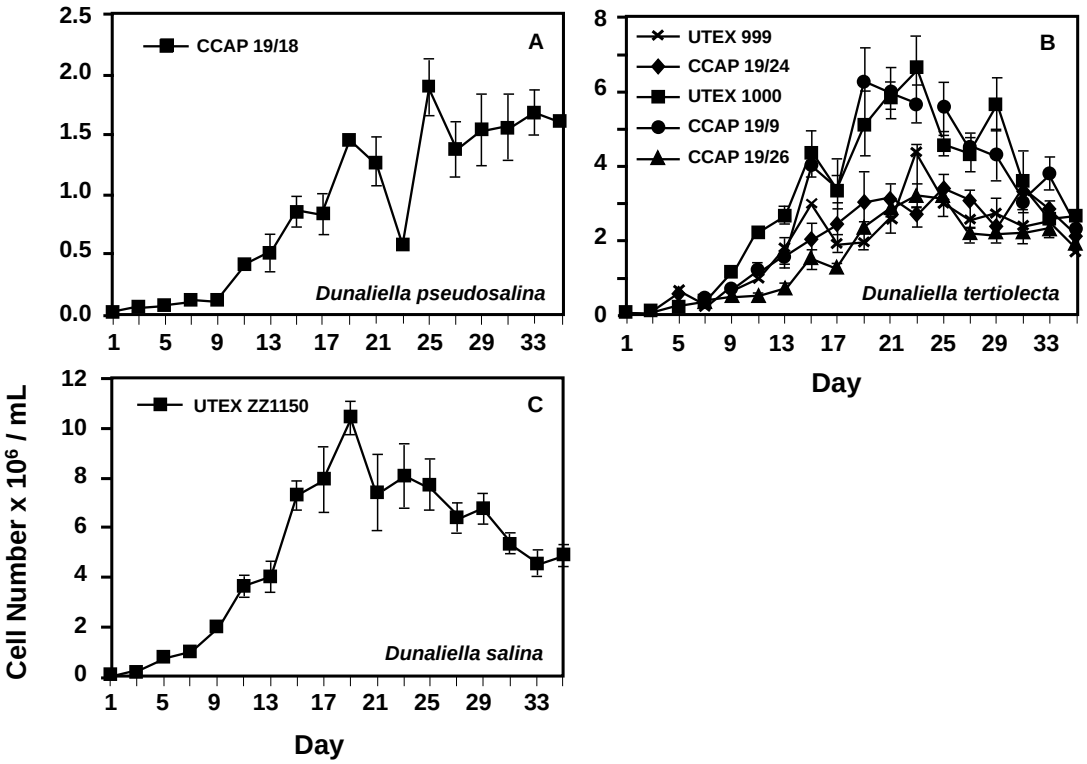
1113
1114 **Fig. 9.** Total free glycerol content in *D. viridis* UTEX ZZ1150 under short-term high-
1115 salinity stress when the control salinity of 60 was increased to 90 - following centrifugation,
1116 total free glycerol A) In the pellets (mg/g); and B) In the supernatant (mg/mL). Note the
1117 change in scale for these two graphs. Significant differences from the control are indicated
1118 by an asterisk ($p \leq 0.05$). Data are given as means ± 1 SE (n = 3).

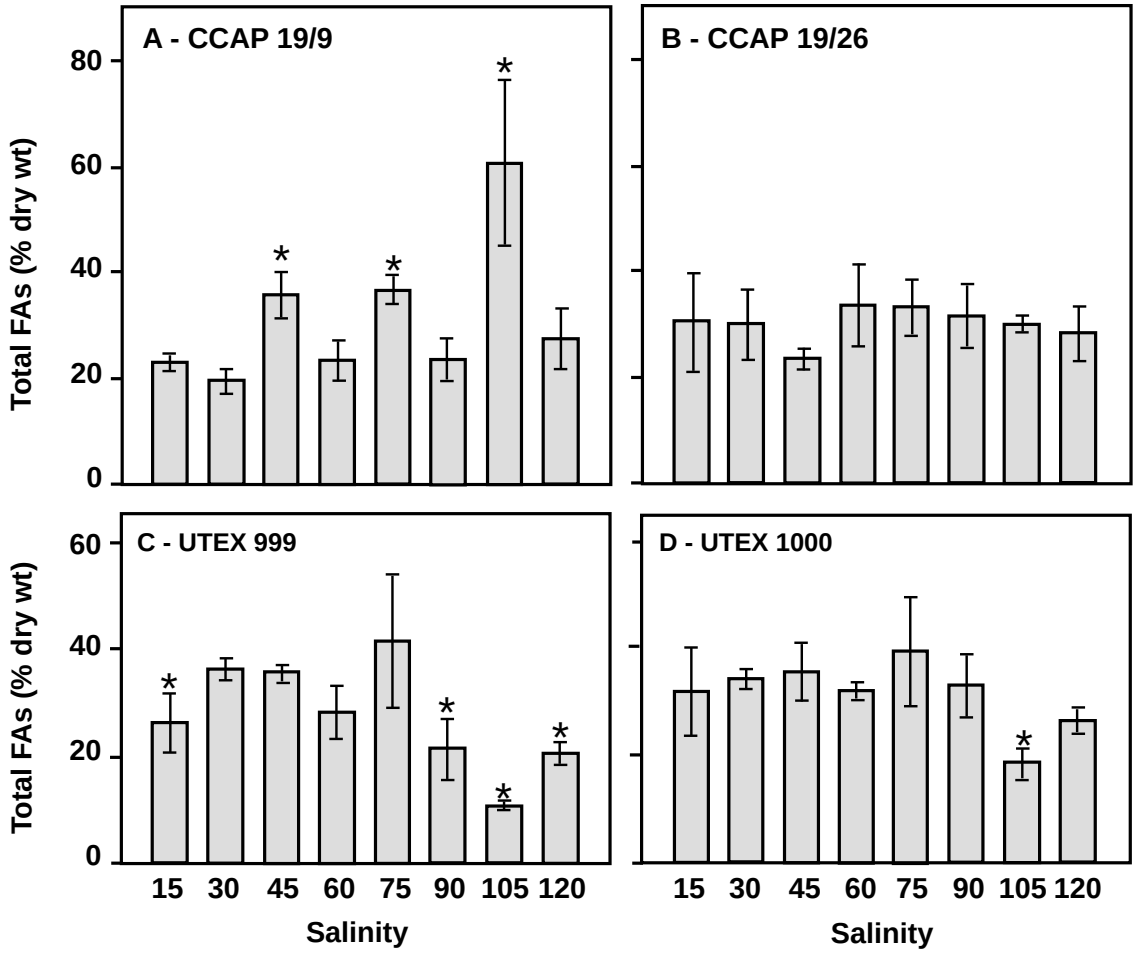
1119
1120 **Fig. 10.** Expression of *GPDH* in *D. viridis* UTEX ZZ1150 under short-term high-salinity
1121 stress when the control salinity was increased to 90. Expression values are given as ratios
1122 relative to the values of *ACTIN*. Data are given as means ± 1 SD (n = 3).

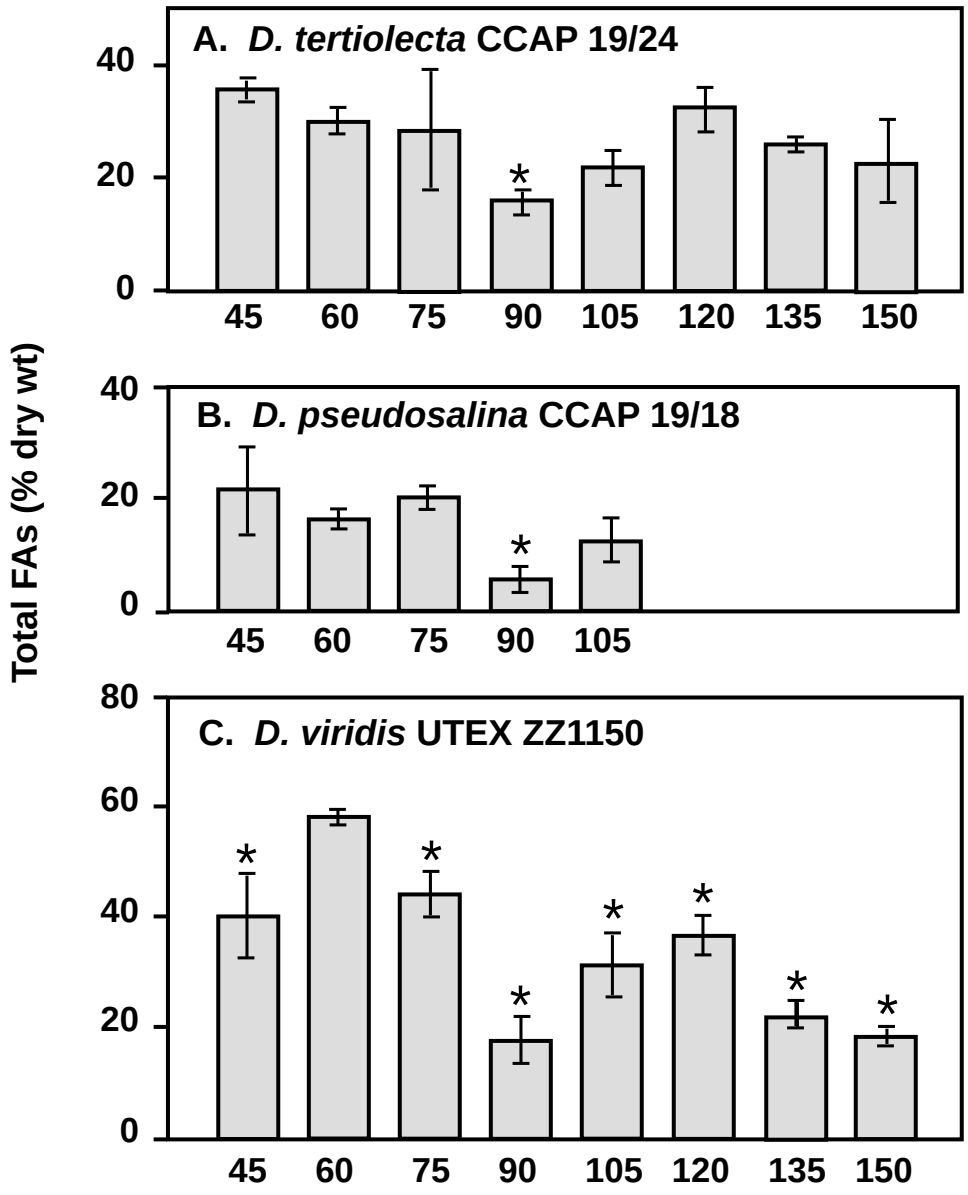

Starch
Sucrose
Glucose 6-P
G6P-1
Fructose 6-P
Chloroplast
PF6P-P
Fructose 1,6-BP
F1,6-A
DHAP
Glycerate 1,3-BP
GPDH
PEP
Glycerol 3-P
Pyruvate
Pi
Cytosol
Pi
DHAP
GPDH
DHA kinase
Glycerol 3-P
DHA
GPI
DHA
reductase
Glycerol

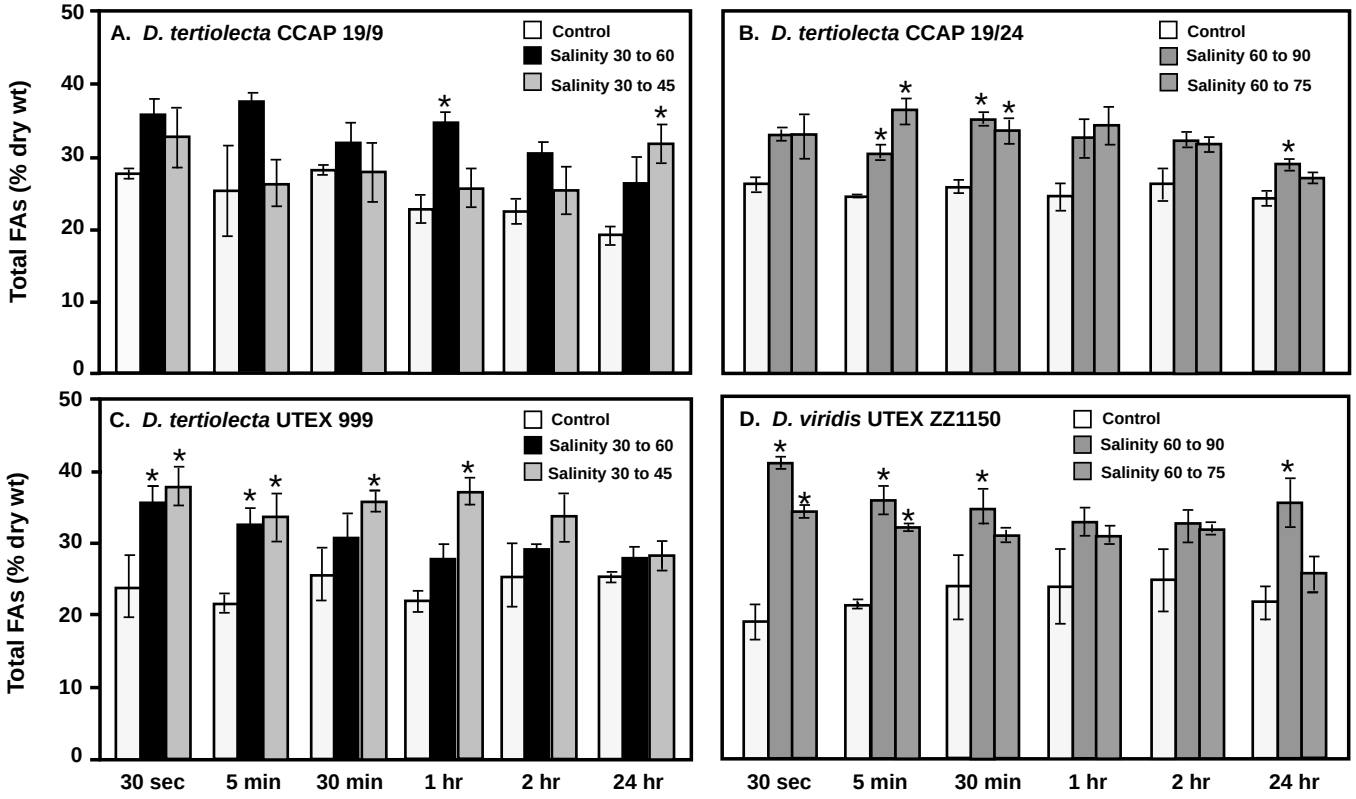

A
B
C
D
E
F
G
H
I
J
K
L
M
N

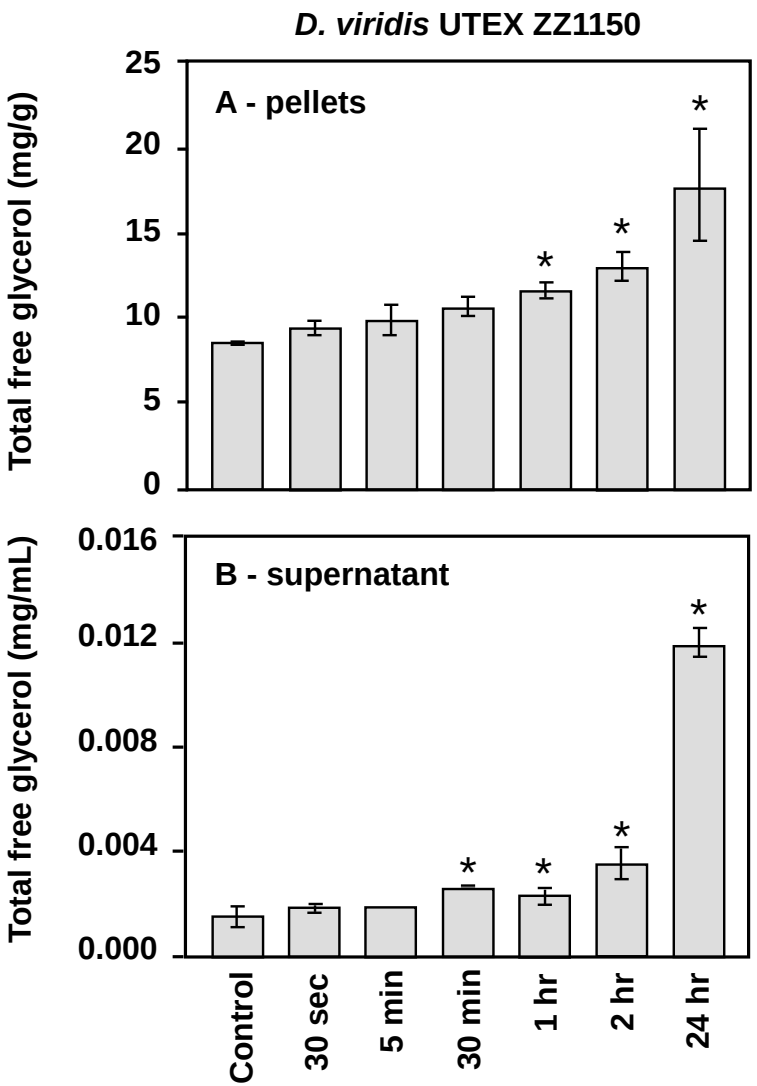

D. tertiolecta CCMP 364
D. parva CCAP 19/26
100
D. tertiolecta UTEX LB 999
D. parva CCAP 19/9
71
D. tertiolecta CCMP 1320
D. tertiolecta CCAP 19/24
D. bardawil ATCC 30861
D. parva CCAP 19/10
100
D. salina CCAP 19/3
D. salina UTEX LB1644
71
D. salina UTEX LB200
D. salina CCAP 19/18
100
D. parva UTEX LB1983
0.02

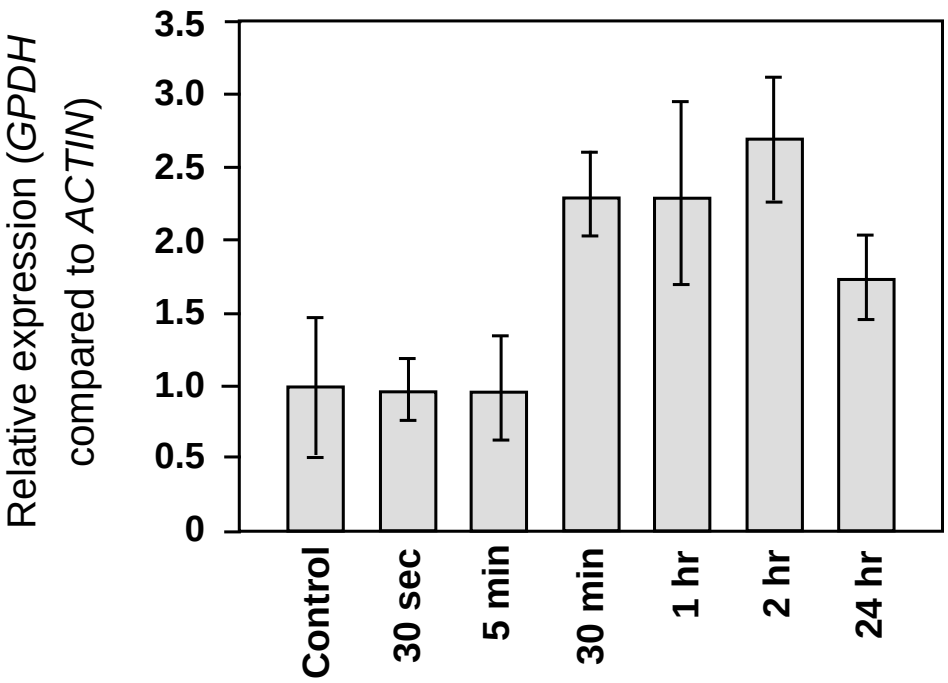

CCAP 19/18
A
Dunaliella pseudosalina
UTEX 999
CCAP 19/24
UTEX 1000
CCAP 19/9
CCAP 19/26
B
Dunaliella tertiolecta
UTEX ZZ1150
C
Dunaliella salina
Cell Number x 10⁶ / mL
Day
Day


A - CCAP 19/9
B - CCAP 19/26
C - UTEX 999
D - UTEX 1000
Total FAs (% dry wt)
Total FAs (% dry wt)
0
20
40
60
80
0
20
40
60
15 30 45 60 75 90 105 120
Salinity
15 30 45 60 75 90 105 120
Salinity


A. D. tertiolecta CCAP 19/24
B. D. pseudosalina CCAP 19/18
C. D. viridis UTEX ZZ1150
Total FAs (% dry wt)
0
20
40
60
80
45
60
75
90
105
120
135
150
*


A. D. tertiolecta CCAP 19/9
Control
Salinity 30 to 60
Salinity 30 to 45
B. D. tertiolecta CCAP 19/24
Control
Salinity 60 to 90
Salinity 60 to 75
C. D. tertiolecta UTEX 999
Control
Salinity 30 to 60
Salinity 30 to 45
D. D. viridis UTEX ZZ1150
Control
Salinity 60 to 90
Salinity 60 to 75
Total FAs (% dry wt)
Total FAs (% dry wt)
0
10
20
30
40
50
30 sec
5 min
30 min
1 hr
2 hr
24 hr


D. viridis UTEX ZZ1150
A - pellets
Total free glycerol (mg/g)
25
20
15
10
5
0
B - supernatant
Total free glycerol (mg/mL)
0.016
0.012
0.008
0.004
0.000
Control
30 sec
5 min
30 min
1 hr
2 hr
24 hr


Relative expression (GPDH compared to ACTIN)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Control
30 sec
5 min
30 min
1 hr
2 hr
24 hr

Species Name (Basis: ITS Sequence Data from this Study	Commercial Source and Strain (Species)	Biovolume (μm^3; n = 30 cells)
D. pseudosalina	CCAP 19/18 (*D. salina*)	215 ± 10
D. pseudosalina	UTEX 1983 (*D. parva*)	240 ± 11
D. pseudosalina	UTEX 200 (*D. salina*)	183 ± 8
D. salina	ATCC 30861 (*D. bardawil*)	646 ± 218
D. tertiolecta	CCAP 19/9 (*D. parva*)	120 ± 6
D. tertiolecta	CCAP 19/24	132 ± 6
D. tertiolecta	CCAP 19/26 (*D. parva*)	120 ± 5
D. tertiolecta	NCMA 364	135 ± 8
D. tertiolecta	NCMA 1320	136 ± 6
D. tertiolecta	UTEX 999	163 ± 9
D. tertiolecta	UTEX 1000 (*D. primolecta*)	168 ± 8
D. viridis	CCAP 19/10 (*D. parva*)	46 ± 2
D. viridis	UTEX ZZ1150 (1644 - *D. salina* contaminant)	74 ± 5

Species and Strain Name (Commercial Collections)	Associated GenBank ITS2 Accession No.
CCAP 19/9 ("*D. parva* ")	KF229737
CCAP 19/26 ("*D. parva* ")	KF229741
CCAP 19/24 *D. tertiolecta*	KF229743
NCMA 364 *D. tertiolecta*	KF229740
NCMA 1320 *D. tertiolecta*	KF229742
UTEX 1000 ("*D. primolecta* ")	KF229739
UTEX 999 *D. tertiolecta*	KF229738
UTEX 1983 ("*D. parva* ")	KF229736
CCAP 19/18 ("*D. salina* ")	KF229735
UTEX 200 ("*D. salina* ")	KF229734
CCAP 19/10 ("*D. parva* ")	KF229731
CCAP 19/3 ("*D. salina* ")	KF229732
UTEX 1644 ("D. salina")	KF229730
ATCC 30861 ("*D. bardawil* ")	KF229733

* Now *D. viridis* strain UTEX ZZ1150.

Clade Determined from ITS Sequence Data	Species and Strain Number Given by Commercial Source
Dunaliella pseudosalina	(*D. salina* CCAP 19/18)
Dunaliella pseudosalina	(*D. salina* UTEX 200)
Dunaliella pseudosalina	(*D. parva* UTEX 1983)
Dunaliella salina	(*D. bardawil* ATCC 30861)
Dunaliella tertiolecta	(*D. parva* CCAP 19/9)
Dunaliella tertiolecta	(*D. tertiolecta* CCAP 19/24)
Dunaliella tertiolecta	(*D parva* CCAP 19/26)
Dunaliella tertiolecta	(*D. tertiolecta* NCMA 364)
Dunaliella tertiolecta	(*D. tertiolecta* NCMA 1320)
Dunaliella tertiolecta	(*D. tertiolecta* UTEX 999)
Dunaliella tertiolecta	(*D. primolecta* UTEX 1000)
Dunaliella viridis	(*D. salina* CCAP 19/3)
Dunaliella viridis	(*D. parva* CCAP 19/10)
Dunaliella viridis	(contaminant of *D. salina* UTEX 1644, now UTEX ZZ1150)

* Experiment was not replicated because preliminary trials showed higher growth was obtained in L1-Si medium.

Primer	Sequence (5′ to 3′)
ACTIN – Forward	TAGCTGTTTGCGTGTGTGTGCT
ACTIN – Reverse	CATCCTGCATTCCTTCCATT
GPDH – Forward	GGCAATGGCATGTGTAGTTG
GPDH – Reverse	TACAGGTCTCCCTGCTCTCG

FA Composition	CCAP 19/9	CCAP 19/26	UTEX 999
C 16:0	17.10 ± 1.30	18.19 ± 0.73	15.66 ± 0.52
C 16:1, cis9	5.30 ± 0.11	5.03 ± 0.22	4.66 ± 0.04
C 16:3, cis	14.03 ± 0.11	12.85 ± 0.61	12.15 ± 0.11
C 17:0	na	na	na
C 18:1, cis9	5.58 ± 0.71	8.70 ± 1.63	8.85 ± 0.64
C 18:2, cis9,12	3.84 ± 0.09	5.40 ± 0.87	5.05 ± 0.44
C 18:3, cis6,9,12	3.74 ± 0.07	3.60 ± 0.12	3.72 ± 0.02
C 18:3, cis9,12,15	30.72 ± 0.28	22.25 ± 7.07	27.24 ± 0.46
Unknowns	13.44 ± 0.15	9.37 ± 1.25	11.63 ± 0.83

Strain	Final Salinity	FAs (mean ± 1 SE)	LS Mean Diff
D. tertiolecta CCAP 19/9	15	23.01 ± 1.57	3.39
	30	19.61 ± 2.67	na
	45	35.88 ± 4.32	16.27
	60	23.25 ± 3.57	3.64
	75	36.44 ± 2.62	16.83
	90	23.56 ± 4.07	3.95
	105	60.64 ± 15.53	41.03
	120	27.40 ± 5.70	7.79
D. tertiolecta CCAP 19/24	45	35.72 ± 1.71	5.53
	60	30.19 ± 2.14	na
	75	28.75 ± 10.92	−1.45
	90	15.87 ± 2.12	−14.30
	105	22.08 ± 3.05	−8.11
	120	32.36 ± 3.84	2.17
	135	26.07 ± 0.95	−4.13
	150	22.90 ± 7.08	−7.29
D. tertiolecta CCAP 19/26	15	30.29 ± 9.22	0.61
	30	29.69 ± 6.61	na
	45	23.44 ± 1.97	−6.25
	60	33.38 ± 7.62	3.69
	75	32.99 ± 5.10	3.30
	90	31.47 ± 5.88	1.78
	105	29.87 ± 1.67	0.19
	120	28.02 ± 4.66	−1.67
D. tertiolecta UTEX 999	15	26.30 ± 5.41	−10.10
	30	36.38 ± 1.98	na
	45	35.73 ± 1.45	−0.65
	60	28.33 ± 4.95	−8.05
	75	41.66 ± 12.38	5.28
	90	21.56 ± 5.58	−14.80
	105	10.71 ± 0.68	−25.70
	120	20.78 ± 1.95	−7.29
D. tertiolecta UTEX 1000	15	31.69 ± 8.15	−2.08
	30	33.77 ± 1.78	na
	45	35.26 ± 5.15	1.49

Strain	Salinity	Time Point	Mean FAs ± 1 SE
D. tertiolecta CCAP 19/9	Control	30 sec	27.42 ± 0.64
		5 min	25.23 ± 6.34
		30 min	28.27 ± 0.58
		1 hr	22.89 ± 1.80
		2 hr	22.29 ± 1.66
		24 hr	19.14 ± 1.22
	30 to 45	30 sec	32.83 ± 3.55
		5 min	26.20 ± 2.94
		30 min	27.79 ± 3.51
		1 hr	25.75 ± 2.36
		2 hr	25.40 ± 2.87
		24 hr	31.69 ± 2.18
	30 to 60	Before	29.06 ± 1.61
		30 sec	35.94 ± 1.74
		5 min	37.83 ± 1.13
		30 min	31.95 ± 2.42
		1 hr	34.87 ± 1.12
		2 hr	30.54 ± 1.37
		24 hr	26.55 ± 3.14
D. tertiolecta CCAP 19/24	Control	30 sec	26.18 ± 0.92
		5 min	24.41 ± 0.10
		30 min	25.87 ± 0.92
		1 hr	24.52 ± 1.80
		2 hr	26.20 ± 2.40
		24 hr	24.15 ± 1.04
	60 to 75	30 sec	32.96 ± 2.54
		5 min	36.36 ± 1.46
		30 min	33.31 ± 1.53
		1 hr	34.34 ± 2.28
		2 hr	31.61 ± 0.97
		24 hr	26.98 ± 0.54
	60 to 90	30 sec	33.05 ± 0.68
		5 min	31.02 ± 1.03
		30 min	35.52 ± 0.67